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EXPERIMENTAL STREPTOCOCCUS EMPYEMA

II. ATTEMPTS AT DYE THERAPY

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Silva
In a previous communication, Gay and Stone¹ have described an experimental syndrome in rabbits produced by streptococcus pyogenes and resembling in all essential points human streptococcus empyema. This experimental empyema can be produced invariably under conditions that will be repeated presently and serves as an admirable localized infection on which to work out the possibilities of prevention and therapy. In our previous report we succeeded, though with considerable difficulty and danger to the animal, in producing an active immunity by means of vaccines. Therapy by the use of serum from actively protected animals and containing various immune bodies was successful in only a few instances, although in view of the questionable value of the results obtained by means of vaccine and serum it was judged wise as a next step to investigate the possibilities of chemotherapy with dyestuffs.

Much work has been done concerning the influence of dyestuffs on bacteria and for different purposes, and although a comprehensive review of this subject does not here concern us, it may be serviceable to endeavor to point out the general purposes and results that have been obtained. It was early shown by the work of Rozsahegyi,² Stilling,³ and Koch,⁴ that various microorganisms, including the tubercle bacillus, are readily inhibited in their growth (bacteriostasis), or killed by considerable dilutions of dyestuffs, and it was early realized that dye stuffs vary enormously in their bactericidal properties. On the other hand, bacteria were found to vary equally widely in their susceptibility to any particular dyestuff.

The varying susceptibility of bacteria led to the employment of dye stuffs in culture medium for the purpose of isolation and differentiation of certain less susceptible organisms. This was first pointed out by Rozsahegyi² and made use of in a practical way in the isolation of typhoid bacillus by Drigalski and Conradi.⁵

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¹ Jour. Infect. Dis., 1920, 26, p. 265.

² Centralbl. f. Bakteriol., 1887, 2, p. 418.

³ Ibid., 1890, 8, p. 155.

⁴ Trans. Tenth. Int. Med. Cong., 1890.

⁵ Ztschr. f. Hyg. u. Infektionskr., 1902, 39, p. 283.

ABALINOLIN STAINING METHOD

Much work has been done in an attempt to correlate the chemical structure of dye stuffs with their degree of bactericidal action and, on the other hand, to show the group relationships of bacteria in accordance with their dye susceptibilities. The significance of the latter attempts was focused by the work of Churchman,⁶ who showed the definite relationship between the Gram stain and susceptibility to dye action. Subsequently it was indicated that Gram positive micro-organisms are more susceptible than Gram negative bacteria, not only to stains of the para-rosanalin series which are used in this staining method, but also to other groups of dyestuffs as well.

The obvious, practical utilization of these studies, interesting as they are in themselves, lies in the treatment of bacterial infections in the animal body. It may be frankly stated at the outset that we have little assurance at the present time that the interesting results obtained by the action of dyestuffs on bacteria in the test tube may be transferred to the combating of bacterial infections. A number of factors intervene, many of which are clearly understood while others are still in doubt, that interfere with the utilization of dye stuffs therapeutically. It concerns us more particularly to discuss the various factors that intervene and that, in some instances at least, make dye therapy impossible.

Having demonstrated that one of several dye stuffs are bacteriostatic or bactericidal for the kind of micro-organism under consideration in the test tube, we must obviously first consider whether this particular substance can be used in the animal body in view of its degree of toxicity for animal tissues; in other words, the criterion established by Ehrlich for chemotherapeutic tests must be followed. The substance to be used must be, so far as possible, monotropic, uniting readily with the bacterial protoplasm and not with the protoplasm of the host. The actual physical condition of any kind of dyestuff will be found to influence its toxicity markedly; for example, certain conditions of dilution and heating may render a nontoxic dye colloidal and highly toxic.

Let us suppose that a certain dyestuff is relatively nontoxic for the host and highly bactericidal, we must then assume that it undergoes no essential change until it reaches the bacteria in the body of the host, and we must further be assured that it remains in a condition of simple dilution in the fluids of the animal for a sufficient period of time to assure its complete destructive effect on the bacterial cell. As a matter of fact, a number of things may happen that will prevent

⁶ Jour. Exper. Med., 1912, 15, p. 221; 1917, p. 373, 16, p. 822.

these desiderata. The contact between bacteria and dyestuff in the animal body will approximate the test tube conditions only in the case of localized accessible infections, as in wounds or when the dye stuff is injected into closed cavities, as in the particular empyema we are studying. When the dye is given into the general circulation for the purpose of reaching some more or less remote region the results become still further complicated.

In the last and extreme instance when introduced into the circulation many dyestuffs that are tolerated by the animal and highly bactericidal will at first stain the tissues diffusely but after a few minutes or hours the color will be found to have disappeared. On testing various organs or excretions it may be found that the dye has been simply reduced to its leuco base and its color may be restored by oxydation; or the destruction of the dyestuff may proceed further. In both of these instances the bactericidal effect is apparently lost. And again it may be found that although a dyestuff remains generally unchanged in color, it will become rapidly absorbed by certain tissues of the body and may thereby leave the infected areas free from bactericidal action.

A still further limitation to the action of dyestuffs would lie in the possibility of the bacteria concerned becoming dye fast. A thorough consideration of these and other factors concerned in the application of dyestuffs to infections will concern us in connection with the work herewith presented.

THE BACTERICIDAL EFFECT OF CERTAIN DYESTUFFS IN VITRO

We have made a comparative test of a considerable number of dyestuffs in an attempt to find those most active against the streptococcus. Although not primarily interested in the relation of chemical structure to bactericidal properties or the relation of specific dyestuffs to certain bacteria, we have incidentally studied the bactericidal effects of the dyes under investigation, not only on the streptococcus but also on *B. typhosus* and *staphylococcus*. Certain general observations may be made from our results which may be of interest apart from the specific action of these dyes on streptococcus.

Considerable differences in the results of various investigators have probably arisen from differences of technic and in many instances the particular technic in bactericidal experiments with dyestuffs has

not been given. The technic would vary with the object in view. In our own experiments, dealing as we have with a massive and localized infection, we have utilized large numbers of bacteria in every test. The standard technic on which our results are based consisted in preparing a series of tubes of dyestuff in successive dilutions from 0.1% to the eventually found limit of activity. The dyes were first made up in 1% solution in sterile distilled water and subsequent dilutions made from them by adding beef infusion broth of a P_H of 7.2 to 7.5. With all the active bactericidal dyes, and indeed, with most of the others, such dilutions were found to be perfectly sterile. In a few instances dyes were autoclaved after dilution, with variable results as regards their bactericidal properties. To a series of dilutions was added an equal amount (1 c c) of 24-hour broth culture of streptococcus "H" or the other organisms to be mentioned. The tubes were then incubated for 24 hours and streaks on blood-agar plates were made to test viability. In most instances this arbitrary limit of 24 hours was found to give the ultimate degree of bactericidal property, although in some cases further incubation for another 24 hours led to sterility in a higher dilution. In a number of instances it was found, moreover, that the limit of bactericidal property was the same irrespective of the number of organisms introduced, at least within the limits of from 1 c c as ordinarily used to 0.1 c c of the same culture.

A brief statement of the bactericidal properties of the dyestuffs we have tested in respect to the various micro-organisms under consideration follows:

1. The following dyes[†] were not bactericidal for streptococcus "H," for staphylococcus aureus 112, or for *B. typhosus* 3 in a final dilution of 1:2000:

Benzo-azurin (H)	Congo red (J)
Azo-acid red (N)	Cyanin B. (M)
Diamil blue (J)	Oxamin violet (B)
Erioglaucin A. (G)	Sauer grün (J)
Acid fuchsin (J)	Scarlet 6 R. (M)

2. Acid violet, although inactive, at the dilution of 1:2000, for *B. typhosus* and streptococcus was found bactericidal for staphylococcus at 1:20,000.

[†] We are indebted to Prof. H. M. Evans for many of the dyes employed and for information concerning them. The dyes were from the following manufacturers as indicated by the letter following the name:

A., Berlin Anilin Works.
B., Badische Co.
By., Bayer Co.
C., Cassella.
D., A. H. Thomas Co.
E., Eimer and Ahmend Co.
F., Hynson, Westcott and Dunning.
G., Geizy.

H., Harmer Lab. Co.
I., Boots Co.
J., Grüber.
L., A. Leonhardt & Co.
M., Meister, Lucius and Bruening, Ltd.
N., H. A. Metz.
O., Elberfeld.
P., Society of Chemical Industry, Basle.

3. The following dyes were found bactericidal for streptococcus, but not for staphylococcus and *B. typhosus* at a dilution of 1:2000:

Acridine orange (L)	Neutral red (J)
Brilliant cresyl blue (H)	Safranin (H)
Columbia blue R. (A)	Sulphon acid blue R. (O)
Crystal ponceau (A)	Wasser blau (B)
Methylene blue (med. pure) (J)	Toluidin blue (J)
Methylene blue (G. G.) (C)	Janus dark blue B (M)
Nile blue (By)	

4. The following dyes were found bactericidal for streptococcus and staphylococcus but not for *B. typhosus* at a dilution of 1:2000 or more:

Basic fuchsin (J)	Rhodulin violet (By)
New fast green 3 B. (P)	Setocyanin (G)

5. The following dyes were found bactericidal for streptococcus, *B. typhosus* and for the most part for staphylococcus at dilutions of 1:2000 or more, as expressed comparatively in table 1.

TABLE 1
DYES BACTERICIDAL FOR STREPTOCOCCUS, STAPHYLOCOCCUS AND *B. TYPHOSUS*

	Streptococcus	Staphylococcus	<i>B. Typhosus</i>
Auramine (E).....	2,000	0	2,000
Methylene green (B).....	2,000	0	2,000
Acridine (F).....	20,000	20,000	20,000
Janus black "2" (M).....	20,000	0	2,000
Crystal violet (A).....	200,000	20,000	20,000
Proflavine (I).....	200,000	20,000	20,000
Spiller's purple (J).....	200,000	20,000	2,000
Janus green (M).....	200,000	2,000	2,000
Brilliant green (D).....	2,000,000	20,000	2,000
Malachite green (A).....	2,000,000	20,000	2,000
Methyl violet (H).....	2,000,000	20,000	2,000
Solid grün (J).....	2,000,000	20,000	2,000

Some general conclusions would seem warranted from the results obtained. In a general way both streptococcus and staphylococcus are more susceptible to dyestuffs, with few exceptions, than is *B. typhosus*. Generally speaking, the streptococcus studied is more susceptible than the strain of staphylococcus. These suggestions may possibly be offered: It would seem that diazo dyes have relatively little bactericidal properties. The most active dyes for streptococcus belong to the triphenylmethane and acridine group.

At this point may be mentioned certain other test tube experiments we have undertaken as bearing on certain limitations of dye action. It would be suggested from the work on the chemotherapy of protozoan infections that an inefficiency of dyes on bacteria might exist if the latter were able to acquire gradually "dye fastness." Browning and Gulbransen⁸ have actually tested for fastness with flavine and brilliant

⁸ British Med. Jour., 1917, 1, p. 73.

green and found no indication of its existence. Simon and Wood,⁹ however, have claimed that dye fastness may arise. Although our experiments are not extensive on this point, they are unequivocal and show clearly that repeated subcultures of the streptococcus in the highest nonbactericidal dilution of several of the most active dyes (acriflavine, new fast green 3 B, safranine and malachite green) not only did not render the micro-organisms more resistant to more concentrated solutions of the dye, but also actually seem to develop a greater susceptibility to the dye in dilutions in which it originally grew.

Several experiments were undertaken to consider the possibilities of the mixture of bactericidal dyes. It was thought possible that a combination of dyes might be more bactericidal than either of them alone. It was found, however, that the final bactericidal dilution of such mixed dye solutions might reach the limit of the stronger dye, but in case of interaction and precipitation between the two solutions the bactericidal effect was actually decreased.

As we have already mentioned, one of the recent important advances in the knowledge of the action of dyestuffs on bacteria has been the appreciation that many of them, although active under conditions of artificial cultivation of bacteria in the test tube, are inactive in protein solutions that simulate conditions in the body. The recent advocacy of the use of the flavine or acridine compounds in the treatment of wounds lies in the demonstration by Browning and Gulbranson,⁸ Hewlett,¹⁰ Dakin and Dunham¹¹ and others, that in contradistinction to most dyes these compounds are fully as active or even more active against bacteria in protein solutions than in broth or salt solution. We will take up in connection with the experiments in the living body, the other advantages that are claimed for them, but it was obvious that before proceeding to animal experiments we should test out the various dyestuffs that have been found to be most active in broth solutions against the streptococcus on this micro-organism as it actually occurs in our experimental empyema.

The experimental empyema in rabbits is produced by injecting 0.2 c.c. of a 24-hour broth culture of a passage strain of *Streptococcus pyogenes* "H" into the right pleural cavity. The passage strain is conserved in the pleural fluid derived from previous cases which may be kept in the icebox without deterioration for several weeks. Although such pleural fluids become sterile at room

⁹ Amer. Jour. Med. Sci., 1914, 147, p. 524.

¹⁰ Lancet, 1917, 193, p. 727.

¹¹ Handbook of Antiseptics, 1918.

or incubator temperature in a few days, the number of streptococci increases gradually in the icebox for about 3 weeks and then gradually decreases although subcultures are positive for at least 2 months. It should further be noted that although the samples of fluid are taken from the infected pleura at necropsy with only moderate precautions and placed in large tubes that are repeatedly opened for subcultures, they have never been found contaminated. This passage culture has assumed fixed virulence so that it succeeds invariably in producing a progressive pleuritis extending from the right side to the left through the pericardium without producing septicemia except in the terminal stages.¹² Infection is fatal in from 3 to 7 days at which period 20 c c to 30 c c of a purulent exudate is found in each pleural cavity.

A series of tests have been made with the dyestuffs that were found active in broth against the streptococcus in dilutions of 1:2000 or better on this pleural fluid derived from fatally infected rabbits. The technic of these experiments was similar to that described for the broth cultures and in each instance 1 c c of pleural fluid was added to the dye dilution. Several estimates of such fluids show that a cubic centimeter contains from 200 million to two billion streptococci as compared with a 24-hour broth culture which on an average contains one billion organisms. A comparative action of the various dye stuffs as against streptococci broth cultures and in pleural fluid is shown in table 2.

TABLE 2

THE BACTERICIDAL TITER OF VARIOUS DYESTUFFS ON *S. PYOGENES* "H" IN:

	Pus	Broth Culture
Buffalo fast crimson R (?).....	0	2,000
Columbia blue R (A).....	0	2,000
Crystal ponceau (A).....	0	2,000
Methylene green (B).....	0	2,000
Nile blue (By).....	0	2,000
Wasser blau (B).....	0	2,000
Sulphon acid blue (?).....	0	20,000
Spiller's purple (J).....	0	200,000
Setocyanin (G).....	0	200,000
Pyronin (E).....	0	200,000
Brilliant cresyl blue (H).....	2,000	20,000
Methylene blue G. G. (C).....	2,000	20,000
Methylene blue (Med. Pur.) (J).....	2,000	200,000
Janus black "2" (M).....	2,000	20,000
Janus dark blue (M).....	2,000	200,000
Neutral red (J).....	2,000	200,000
Toluidine blue (J).....	2,000	200,000
Brilliant green (D).....	2,000	2,000,000
Malachite green (A).....	2,000	2,000,000
Methyl violet (H).....	2,000	2,000,000
Safranin (H).....	2,000	2,000
Methylene green (B).....	2,000	2,000
Acridine orange (L).....	20,000	200,000
Janus green (M).....	20,000	200,000
New fast green 3 B (P).....	20,000	200,000
Basic fuchsin (J).....	20,000	20,000
Rhodulin violet (By).....	20,000	20,000
Solid grün (J).....	200,000	2,000,000
Acridine orange (F).....	200,000	20,000
Proflavine (I).....	200,000	200,000

¹² Twenty-seven normal controls have been positive; of 85 other animals treated in various ways in an attempt to modify conditions only 2 have survived, and these were treated with an immune serum. There may be contrasted with these figures, actively immunized animals which, as shown in the previous report, are fully protected.

Most of the dyes studied lose markedly in bacterial power in the presence of pus. A considerable number remain rather active and a few of them, safranin, methylene green, basic fuchsin, rhoduline violet and the two flavine compounds are as active in pus as in broth. Acriflavine, as in Browning's⁶ experiments with serum, is actually more active in pus than in broth.

THE ACTION OF SELECTED DYES ON EXPERIMENTAL EMPYEMA

We have carried out series of experiments on experimental empyema in rabbits with certain of the dyestuffs chosen from among those that were found active in vitro on streptococcus pus from this condition. With 5 of these dyes—janus green, solid green and safranin, methylene blue (med. pure) and new fast green 3 B—our observations were merely casual and were not continued because no encouraging results were obtained. These experiments may be briefly summarized:

1. *Exper. with Safranin.*—Rabbit 17, weight 1,500, Feb. 16, 1920, was inoculated in the right pleural cavity with 0.2 cc of a 24-hour broth culture of *S. pyogenes* "H" from pleural fluid (such injection which is invariably positive as already noted will hereafter be referred to simply as infection with streptococcus).

On the following day there was injected into the same pleural cavity 5 cc of a 0.5% solution of safranin in normal saline. The animal was found dead on the following day and at necropsy the entire musculature of the thorax showed a faint pink color and the diaphragm was a deep pink color. The urine was distinctly colored. The right pleural cavity contained 26 cc, slightly cloudy, highly colored fluid, containing grumous masses. The left pleural cavity contained 6 cc of the same turbid fluid. Cultures from both cavities were positive for *S. pyogenes*. In this experiment it should be noted that apart from the possible toxicity of the dye for the animal, the dye remained to a large extent localized but did not sterilize. Judging from the amount of fluid obtained in the right pleural cavity there must have been for a period of 24 hours a dilution of the dye stuff of approximately 1:1,000 which may be compared with the bactericidal titer of this dye in pus, of 1:2,000.

2. *Exper. with Janus Green.*—Rabbit 46, weight 1,250, was infected on May 11 with streptococcus and on May 12 injected into each pleural cavity 1 cc of a 0.1% solution of janus green B. The animal was found dead on the following day and at necropsy presented these findings: The right pleural cavity contained 12 cc of fibrinopurulent pus of a light magenta shade. (It should be noted that janus green is a derivative of safranine, according to Green¹³). The left cavity contained fluid of the same shade. The organs adjacent to the diaphragm were all stained with the same magenta color. Cultures from both right and left cavity were positive for streptococcus.

In this experiment, although the dyestuff was apparently reduced, it should be noted that the dilution of the dye in the right cavity was approximately

¹³ Organic Coloring Matters, 1908.

1:1,200 and in left cavity 1:400, whereas the bactericidal titer of this dye in pus had been found to be 1:2,000.

3. *Exper. with Solid Green*.—Rabbit 48, weight 1,200, was infected May 11, 1920. On the following day the animal received 1 c c of 0.1% solution of solid green in each pleural cavity. It was found dead on the following day and at necropsy the right cavity contained 3 c c of fibrinopurulent pus of light green color. The lung was apparently in good condition. The left cavity contained 1.5 c c of clear green fluid. The base of the left lung was consolidated. The cortex of the kidneys was stained green. The urine was normal in appearance. Cultures from the right pleural cavity were positive; cultures from the left, which was the last to be invaded, proved sterile. In this cavity the concentration of dyestuff equals 1:1,500. In the right cavity destruction of the organism was not accomplished in dilution of 1:3,000. The bactericidal titer in vitro of this dye in pus proved to be 1:200,000. No apparent disintegration of the dye had taken place.

Rabbit 53, weight 1,500, was infected May 18, 1920, and 6 hours later it received 1 c c of 0.5% solid green solution in each pleural cavity. The animal was found dead the following day, and the right cavity contained 5 c c of green pus with a small amount of fibrin. Pericarditis was present. The left pleural cavity contained 2 c c of bloody fluid. Cultures of both cavities were positive. In this case the presumed dilution of dye was 1:1,000.

4. *Exper. with Methylene Blue*.—Rabbit 317, weight 2,500, was infected April 20, 1920, and the following day received 2 c c of a 1% solution of methylene blue (med. pure Grüber) in each cavity. The animal was found dead the following day. The hair about the external genitals and the urine was found stained a bright blue. Six c c of bright blue pus was found in the right pleural cavity. The lung was congested and covered with a fibrinous exudate. The left cavity contained 6 c c of a turbid slate colored fluid. Cultures from both cavities were positive for streptococcus. The dilution of dye in this cavity was 1:300, whereas the bactericidal pus titer as tested in vitro was 1:2,000.

5. *Exper. with New Fast Green 3 B*.—Rabbit 40, weight 1,900, was infected April 6, 1920. On the following day, it was given 2 c c of the supernatant fluid of a 0.5% solution and suspension of new fast green 3 B in sterile salt solution at room temperature.¹⁴ Twenty-four hours later the right pleural cavity was aspirated and 9 c c of pus of a brownish color removed. Positive cultures were obtained from this pus. On the next day, that is, 4 days after infection, the animal was found dead. The right pleural cavity contained 4 c c of bloody turbid fluid, the left cavity 5.5 c c of the same. No green coloration was evident except about the inferior vena cava and the musculature and cartilage of the trachea. Positive cultures from both cavities were obtained. In this instance the original dilution of dye, which apparently lost its color in pus, was in a concentration of 1:1,000, whereas the bactericidal titer outside the body had been found to be 1:20,000.

Experiments with Acriflavine.—Our experiments with the acridine compounds, particularly the one known as acriflavine, are more exten-

¹⁴ It should be stated here that new fast green 3B is completely soluble in water or salt solution in about 1/16 of 1%. In efforts to dissolve larger amounts of this dye, higher temperatures have been resorted to with the result that although the dye gives perfect solution while warm and may subsequently remain fluid at body temperature, it jells on cooling. Coincidentally with this colloidal suspension of the dye, a striking increase in its toxicity appears as well as its ability to stain the animal body diffusely. Under the conditions mentioned in this protocol the dye is apparently not toxic and does not stain diffusely.

sive and would seem to have exhausted the logical possibilities for the use of this dye in treatment of experimental streptococcus empyema. As we have already indicated, work in recent years has tended to bring these dyestuffs into prominence because they have been found not only highly bactericidal in broth and salt solution, but also in protein mixtures (Browning⁶ and Hewlett¹⁰). Our own experiments and those of Dakin and Dunham¹¹ have confirmed these observations. It is further asserted by Browning and his collaborators that acriflavine does not inhibit phagocytosis, that it penetrates tissues, and gives rise to no "dye fastness" in bacteria. Our experiments in the living animal have been carried out entirely with acriflavine, owing to the fact that it is somewhat more bactericidal in pus solutions than proflavine as judged from our own work and from that of Dakin and Dunham. Browning and Gulbransen,¹⁵ moreover, have tested these substances in mice and found acriflavine somewhat less toxic than proflavine, although it was judged that both could be used effectively in tolerable doses. Although Browning and Gulbransen found that pneumococcus infections in mice could be inhibited by acriflavine, living organisms still remained in certain tissues. The actual results obtained from the use of these substances in infections we shall refer to more fully in commenting on our own results. The only objection that has been raised to the possible effectiveness of acriflavine compounds is that they are somewhat slow in action. It is indicated from the work of Browning and his collaborators and from our own work also on acriflavine, that it does not become changed in the body although it has an elective affinity for muscle. The following examples are chosen from an extensive series of animals as illustrative of the successive phases through which our work has passed and the characteristic results obtained.

6. *Exper. Illustrating the Tolerance of the Normal Rabbit for Acriflavine.*—Rabbit 13, weight 1,300, received 5 cc of a 0.1% solution of acriflavine in sterile salt solution in the right pleural cavity. There were no symptoms. The dose was repeated on the next day and on the following day the animal was killed and examined. The intestine and stomach were not colored, the urine was stained a deep yellow. The walls and musculature of the right pleural cavity were stained faintly yellow, as was also a persistent thymus. A small amount of clear fluid was found in the left pleural cavity. The right lung had a few spots of atelectasis, otherwise nothing abnormal was noted.

7. *Exper. Showing Action of Acriflavine in Empyema.*—Rabbit B, weight 2,750, was infected with streptococcus pyogenes on Jan. 30, 1920. Three days

¹⁵ Jour. Hygiene, 1919, 18, p. 33; Jour. Path. and Bacteriol., 1919, 22, p. 256.

later it received 5 c c of a 0.5% solution of acriflavine, warmed to body temperature, in the same pleural cavity. On the following day the animal was exsanguinated and necropsy performed. There was no coloring of the abdominal viscera. The pectoral muscles were stained yellow. The right pleural cavity contained 33 c c of light straw colored fluid, which was much less turbid than in untreated animals. Masses of purulent fibrin were found on both visceral and parietal pleura. The left cavity contained 9 c c of a more turbid but clear yellow fluid. There was a marked fibrinous pericarditis. Smears from the pleural fluid showed many phagocytes with streptococci and with no extracellular organisms. Cultures from both left and right cavities were positive.

It should be noted in this instance that the concentration of acriflavine in the right pleural cavity was approximately 1:1,200, whereas the bactericidal titer of this dye in pus had been found to be 1:200,000. Twenty-four hours later, as found at necropsy, the fluid in the right pleural cavity was tested and found to resemble in color a dilution of acriflavine of from 1:10,000. In spite of these facts, the cavity had not become sterilized. It seemed possible that the dye had been given too late to be effective.

Exper. 8.—Rabbit A, weight 2,650, was infected as usual on Jan. 30, 1920, and on the following day given 5 c c of 1% acriflavine in sterile salt solution in the same pleural cavity. The animal showed symptoms of irritation after injection. Death occurred on the following day, and at necropsy the following findings were obtained. The muscles of the thorax and abdomen were stained yellow. There was considerable turbid peritoneal fluid. The right pleural cavity contained 20 c c of reddish brown turbid fluid. The right lung was consolidated at the base and showed adhesions to the thoracic wall. The left pleural cavity contained 10 c c of brightly colored fluid. Cultures from the right pleural cavity were positive in spite of the fact that the dilution of acriflavine must have approximated 1:400. The left pleural cavity was sterile, which may have been due to the failure of the infection to extend to this point, but which suggested that an actual sterilization had occurred.

Exper. 9.—Rabbit 1, weight 2,400, was infected on Feb. 4. The treatment differed from preceding experiments in that 5 c c of the 0.5% of acriflavine solution were given 24 and again 48 hours after inoculation. The animal was found dead 3 days later and at necropsy showed an extensive double pleurisy with 25 c c of fluid in each pleural cavity, which had lost any distinctive yellow color. Cultures from both pleural cavities were positive.

It was evident in this experiment that whatever may have been the temporary action of two relatively large doses of acriflavine, the result was unsuccessful so far as ultimate sterilization was concerned. The animal, moreover, apparently did not die of the action of acriflavine but showed the normal course of an untreated infection, namely, 5 days.

Exper. 10.—Rabbit 3, weight 2,300, was infected as usual and given a single dose of 5 c c of 0.5% acriflavine. The animal was found dead 3 days later and at necropsy 25 to 30 c c of yellow fluid was found in each pleural cavity, which was very clear and not as turbid as in other animals. Cultures taken immediately from this fluid were positive, but when cultures were made 2 days later from the same fluid preserved in the icebox, it was found that the right pleural fluid gave a negative culture and the left showed only a few organisms.

This experiment suggested the possibility that the failure to sterilize empyema cavities in rabbits might be due to a failure of the dyestuff to

work in cavities relatively free from oxygen, as the fluids outside the body in contact with the air became sterile. It should be reiterated that the normal fluids remain viable for many days. This result was obtained in several other experiments. In order to settle the question, dilutions of acriflavine in broth of from 1:1,000 to 1:1,000,000 were made in a series of Hall's¹⁶ anaerobic tubes with marble device. It should be stated that autoclaving has no effect on the bactericidal effect of acriflavine. These various dilutions were inoculated immediately on cooling both above and below the marble with 0.5 cc of a plain broth culture of streptococcus pyogenes "H," that is to say, they were inoculated both anaerobically and aerobically. The cultures were observed for 3 days and streaked on blood-agar plates finally from the portions above and below the marble. In both instances the cultures at 1:100,000 were sterile, whereas those at 1:1,000,000 were not. In other words, the dye-stuff apparently is as effective in the absence of oxygen, and such absence of oxygen would not account for the failure of the dye to work as expected in the pleural cavity.

Exper. 11.—Rabbit 11, weight 1,800, was infected Feb. 10. On Feb. 12 it received 5 cc 0.5% acriflavine and on the following day 2 cc 0.5% acriflavine in the right pleural cavity. The animal was found dead on the following day. Kidneys and urine were highly colored. The right pleural cavity contained 6 cc of bloody fluid. The lung was consolidated. There was no pericarditis. Little fluid appeared in the left pleural cavity; the apex of that lung was also consolidated. Cultures from the right pleural fluid showed only 3 colonies of streptococcus and from the left the fluid was sterile.

This experiment illustrates that although the acriflavine was pushed to the point of almost completely sterilizing the cavities, recovery did not take place.

Exper. 12.—Rabbit 51, weight 2,200, was infected on May 18 as usual, and on the afternoon of the same day it received 1 cc of a 0.5% solution of acriflavine in each pleural cavity. On the following day both cavities were aspirated 23 cc of straw colored fluid were taken from the right pleural cavity and 22.5 cc from the left. Cultures from both were sterile. The dose of acriflavine was repeated after aspiration and the following day aspiration was again practiced with a removal of 15 cc from each cavity. A culture from the right cavity was negative, from the left positive. The animal died the following day and at necropsy showed slight infection about the point of inoculation. There were adhesions and advance fibrinopurulent exudate in both cavities with about 8 cc of fluid in each. Cultures from both fluids were positive. All other organs appeared normal.

This experiment illustrates the termination of what seemed to us the logical possibilities of utilizing acriflavine alone in sterilizing rabbits suffering from experimental empyema. Not only were doses given resulting in a greater concentration of the dyestuff in the pleural fluid than was theoretically necessary to sterilize the pus present within 24 hours, but it was shown that the dye was not decomposed in the body during this period of time although it had been absorbed to some extent by other tissues, particularly by the muscles. As testing

¹⁶ Univ. Calif. Pub. Path., 1915, 2, p. 147.

further possibilities, the injections of dyestuff were repeated and in order to remove mechanically large numbers of organisms and relieve respiratory distress, aspiration was practiced several times. Although we have apparently nearly sterilized the cavity, regrowth of the organism has always occurred. In no instance has the life of the animal been prolonged even with changes in the culture findings. The thought naturally arises that the dyestuff does not penetrate sufficiently into the exudate, which has invariably been found on the pleural surfaces, to kill all bacteria. Every distinct change has been noted in the character of the pleural fluids. They become clearer under the action of acriflavine, the masses of fibrin become smaller and more compact. The fact that these exudates are less turbid suggests that the normal activity of the leukocytes, which may to some extent combat the infection, may have been inhibited. The next step in our investigation was to determine whether this had probably occurred.

THE EFFECT OF ACRIFLAVINE ON PHAGOCYTOSIS AND THE
ADJUVANT ACTION OF IMMUNE SERUM

Browning⁸ has asserted that acriflavine has no inhibiting effect on phagocytosis. It seemed important to investigate this point with the conditions under which we were working and in addition to consider the possibility of employing acriflavine in conjunction with immune serum, which had already been shown to have distinct, although slight effect on our experimental empyema. At our suggestion, Mr. D. D. Stafford undertook a series of experiments which, although largely negative for the point under consideration, in themselves are interesting.

Exper. 13. The Effect of Acriflavine on Phagocytosis and in Sterilizing Cultures in the Presence of Leukocytes.—The essentials of the opsonic technic with streptococcus have already been described in our previous report. Leukocytes were obtained by injecting sterile broth into the peritoneal cavity of the guinea-pig. Streptococcus antiserum had been provided by immunizing rabbits whose active immunity was tested by an intrapleural injection. This serum was inactivated by heating to 56 degrees for ½ hour before employment. The organisms consisted of small amounts of 24-hour broth culture of *S. pyogenes* "H." At the end of 2 hours the tubes were centrifugalized, the supernatant fluid decanted, smears made of the sediment, and opsonic determinations made by counting the percentage of 100 leukocytes that included bacteria. The tubes were continued at 37 C. and streaks made on blood-agar plates to determine the destruction of bacteria at the end of 24 hours. The results of such a test are summarized in the following table.

TABLE 3
RESULTS OF EXPER. 13

Tube	Leukocytes	Immune Serum 1012, 56 C.	Streptococci	Acriflavine	Phagocytosis in 2 Hours, Percentage	Culture After 24 Hours
1	0.2 c c	0.2 c c	0.2 c c	0.2 c c 1:100	16	—*
2	0.2 c c	0.2 c c	0.2 c c	0.2 c c 200	17	—
3	0.2 c c	0.2 c c	0.2 c c	0.2 c c 400	26	—
4	0.2 c c	0.2 c c	0.2 c c	0.2 c c 800	30	—
5	0.2 c c	0.2 c c	0.2 c c	0.2 c c 1600	45	—
6	0.2 c c	0.2 c c	0.2 c c	0.2 c c 3200	44	—
7	0.2 c c	0.2 c c	0.2 c c	0.2 c c 32000	50	—
8	0.2 c c	0.2 c c	0.2 c c	0.2 c c 320000	44	++
9	0.2 c c	0.2 c c	0.2 c c	0.2 c c Saline	42	++
10	0.2 c c	Saline	0.2 c c	0.2 c c Saline	22	++

* The sign (—) means sterile.

This table brings out several interesting points. Although 22% of the leukocytes contain micro-organisms in the control without serum, nearly 50% include them in the tubes in which serum is present. In the more marked concentrations of acriflavine, that is, from 1:800 to 1:100, a distinction of inhibition of phagocytosis has occurred in increasing grade, whereas there is apparently no inhibition of phagocytes in tubes 5 to 8, which actually contain dilutions of acriflavine of 1:6,400 upward. It is of further interest that in the tubes containing serum and leukocytes alone the bacteria, although phagocytized, are not destroyed, whereas up to an actual dilution of 1:128,000 in tube 7, containing sufficient amounts of acriflavine, destruction has occurred. There is a destructive zone, in tubes 5 to 7, in which the acriflavine has apparently not inhibited phagocytosis and has also sterilized the mixture.

Exper. 14.—With the result of the last experiment in mind, several experiments were conducted to determine whether it could be demonstrated that a greater destruction of bacteria in the presence of leukocytes and immune serum with marked phagocytosis occurs than with leukocytes in normal rabbit serum when less opsonic action is evident. Cultures, furthermore, were made both at 24 and 48 hours in such experiments and there was no demonstrable difference in the sterilization titer of acriflavine whether in immune serum, normal serum or salt and irrespective of the presence or absence of leukocytes. In further experiments alexin from the guinea-pig was added for the purpose of possible reactivation of the immune serum. It had, however, no effect on the results as already determined.

There would seem, therefore, little evidence from experiments in the test tube to suppose that combinations of immune serum and acriflavine would work any more satisfactorily in experimental empy-

ema than either one of them alone. In view, however, of the notorious divergence of results obtained in the test tube and the animal body it was judged to test out these conditions in the infection itself, which led to such experiments as are herewith detailed.

Exper. 15.—Rabbit 22, weight 1,700, was infected on March 17, 1920, and on the following day received into the same pleural cavity a mixture composed of 2 c c rabbit antistreptococcus serum 1012 + 0.2 c c of 1% acriflavine. On the following day 10 c c of brownish gray, cloudy fluid was removed and the treatment repeated. Culture from this fluid was positive for streptococcus. On the following day, that is, 3 days after infection, 5 c c of fluid were removed from the right cavity and 2 c c from the left. Cultures from both were positive. The animal died 2 days later with the following findings: The right pleural cavity contained 3 c c of cloudy bloody fluid; the pleural surface was covered with a dirty yellow exudate. About 10 c c of fluid were present in the left pleural cavity with the same amount of exudate. There was slight pericarditis. Cultures from both pleural fluids were positive.

Exper. 16.—Rabbit 28, weight 1,750, was infected on March 24, 1920. On the following day 1 c c of pooled immune rabbit serum containing 0.125% of acriflavine¹⁷ was injected. The animal was found dead on the following day with characteristic findings in both pleural cavities and with positive cultures from their fluid.

DISCUSSION AND CONCLUSIONS

As already stated, there is little certainty of the ultimate usefulness of dyestuffs as disinfectants in actual bacterial infections. This is true not only of generalized infections, in which case dyes would be given into the circulation, but also in the case of localized infections in which their usefulness might seem more probable. In respect to generalized infections, a few references have been given as to theoretical possibilities; Churchman and Herz¹⁸ have stated that the blood of animals that have been injected with considerable amounts of gentian violet retain an increased bactericidal property for about 2 hours. It is known, however, that these dyestuffs are reduced relatively rapidly and are highly toxic. Crystal violet has actually been given intravenously in the treatment of coccidioid granuloma by Brown and Cummins¹⁹ and by Cummins and Sanders²⁰ without definite ill effects but with no distinctive results. In a similar way it has been shown by Browning and Gulbransen²¹ that acriflavine endowed the serum

¹⁷ It had been found in the meantime that greater concentrations of acriflavine than this amount gave a distinct precipitate in the presence of serum.

¹⁸ Jour. Exper. Med., 1913, 18, p. 579.

¹⁹ Arch. Int. Med., 1915, 15, p. 608.

²⁰ Jour. Med. Res., 1916, 35, p. 243.

²¹ Proc. Roy. Soc., 1918, 90, p. 136.

of experimental animals with temporary bactericidal properties, and the use of it has been suggested in the treatment of trench fever with results that are claimed to be favorable (Byam, Dimond, Sorapure, Wilson).²²

The use of dyestuffs in the treatment of wounds and on infected surfaces has been more extensive and was suggested in the earliest work of Stilling,³ who spoke of their possibilities in the treatment of eye infections. Gentian violet has been employed in wounds infected with diphtheria bacillus by Churchman²³ with apparently good results. Brilliant green has been tried by Webb²⁴ and Massie²⁵ with results that may be regarded as indefinite, and the flavine compounds have been employed by a number authors, Drummond and McNee,²⁶ Fleming,²⁷ Pearson²⁸ Carslaw and Templeton²⁹ and Ligat.³⁰ They apparently penetrate to some extent and at least inhibit bacterial growth. They are said to produce rapid granulation.

There is little evidence that any of the dyestuffs have been used successfully in the treatment of closed cavities, except in the work of Churchman,³¹ who treated joint infections by means of lavage with gentian violet solution. Better results in this group of infections, particularly in empyema and in the treatment of carriers, have apparently been reached by the use of hypochlorite solutions. A discussion of this aspect of the problem, however, does not enter within the field we are considering.

Our own results show that the streptococcus is readily destroyed under artificial conditions in the test tube by a large group of dyestuffs, much more readily than the typhoid bacillus and probably also the staphylococcus. Among the most active of these substances are certain dyes of the triphenylmethane series and the acridine compounds. When these dyestuffs are tested for their action on streptococci in the pus of experimental empyema, many of them lose a great part or all of their bactericidal properties. A few dyes, however, have been found which were as active in pus as in broth, among which may

²² Jour. Roy. Army Med. Corps, 1917, 29, p. 560.

²³ Jour. Am. Med. Assn., 1920, 74, p. 145.

²⁴ Jour. Roy. Army Med. Corps., 1918, 31, p. 315.

²⁵ Lancet, 1918, 194, p. 635.

²⁶ Lancet, 1917, 193, p. 640.

²⁷ Lancet, 1917, 193, p. 341.

²⁸ Lancet, 1918, 194, p. 370.

²⁹ Lancet, 1918, 194, p. 634.

³⁰ Brit. Med. Jour., 1917, 1, p. 78.

³¹ Jour. Am. Med. Assn., 1918, 70, p. 1047.

be mentioned safranin, methyl green, basic fuchsin, acriflavine and proflavine.

Several of the dyestuffs found to be active in pus have been tested for therapeutic effects in the experimental streptococcus empyema, which may be produced under proper conditions with uniformity by direct inoculation into rabbits. In no instance was a distinct curative effect produced, owing to various reasons, some of which have been analyzed, particularly in respect to the most favorable of these dyestuffs—acriflavine. Although many of these dyes are more or less toxic for the animal, they can be utilized for direct injection into the pleural cavity in a dosage which should theoretically sterilize. The best of these—acriflavine—given in a dosage many times more concentrated than the bactericidal titer in the test tube, does not lose its color in the animal body for some time, and kills a good many organisms. The pleural fluids often become practically sterile, but apparently become reinfected. There is no evidence that the streptococcus becomes dye fast to acriflavine, but it may be protected in the masses of fibrin that line the pleural cavity. Phagocytosis is inhibited in strong concentrations of acriflavine, such as have usually been employed, but the dye will sterilize in the test tube considerable quantities of pus in a dose which does not inhibit the phagocytic activity of the leukocytes. The failure of acriflavine to act successfully in the pleural cavity is not due to the failure of oxygen. As suggestive results have been obtained by the use of immune serum, combinations of immune serum and acriflavine have been utilized also, but without success in sterilizing the pleural cavity or in prolonging the life of the animal. In a number of experiments repeated treatments with and without the aspiration of the accumulated fluid have also failed.

Our results do not justify anticipation of practical therapeutic results from the use of dyestuffs in empyema due to the streptococcus. It should be added, however, that we have not as yet considered the possibilities of using dyestuffs combined with metallic compounds, such as those with mercury described by Davis, White and Rosen³² and by Young, White and Swartz.³³

³² Jour. Urology, 1918, 2, p. 277.

³³ Jour. Am. Med. Assn., 1919, 73, p. 1483.

ON THE ACTION OF CERTAIN SALTS ON PHAGOCYTOSIS AND VIRULENCE OF STREPTOCOCCI

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That certain salts in about the same concentration as physiologic salt solution may inhibit phagocytosis in vitro was demonstrated by Hektoen and Ruediger,¹ who found that CaCl_2 , BaCl_2 , SrCl_2 , MgCl_2 , K_2SO_4 , NaHCO_3 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, $\text{Na}_2\text{C}_2\text{O}_4$, $\text{K}_4\text{Fe}(\text{CN})_6$, neutralized opsonin in such a way that it did not act on bacteria.

Hamburger and his associates² found that highly diluted solutions of BaCl_2 and SrCl_2 did not stimulate phagocytosis, while MgCl_2 and CaCl_2 had distinctly stimulating effects, especially the latter salt. They studied the phagocytosis of particles of carbon. Eggers³ observed that BaCl_2 and NaF are toxic for leukocytes, while MgCl_2 stimulates phagocytic activity of leukocytes. Neisser and Guerinni⁴ found that a large amount of KI may inhibit phagocytosis and a small amount increase phagocytic activity. Otanni noted that when sodium citrate was added to the blood of tuberculous patients to the extent of 1% the phagocytic action became stronger than that of normal human blood, and Otanni and Shiiba obtained similar results in typhoid fever.⁵ Shiiba demonstrated that in the case of normal blood sodium citrate may neutralize opsonin; hence, in the case of the blood of a typhoid patient the conditions must be different. Maganatsu⁶ noted that a 1% solution of CaCl_2 increases phagocytosis under the influence of immune serum against certain blood corpuscles, but KI in 1% solution has no such effect. Nagai and Ito⁷ found that 0.01-0.005% solution of CaCl_2 given by mouth stimulated the leukocytes of tuberculous patients, but had no effect on the serum.

We see from these reports that different results have been obtained by investigators studying the action of the same salt on phagocytosis. As phagocytosis probably is the most important factor in resistance to streptococcus infection, I undertook to study the influence of different salts on the phagocytosis of streptococci both inside and outside the animal body. In the test tube experiments I used normal rabbit serum and the leukocytes of normal guinea-pig, well washed. The experiments in vivo were made in the peritoneal cavity of the mouse, guinea-pig and rabbit. I observed that 3 hours after the

¹ Jour. Infect. Dis., 1905, 2, p. 135.

² Biochem. Ztschr., 1908, 9, p. 295; 1909, 24, p. 470.

³ Jour. Infect. Dis., 1909, 6, p. 667.

⁴ Matsushita, Parasit. Krankh., 1916, 4, p. 169.

⁵ Saikingaku. Zasshi, 1917, 1918, 1919, 1920.

⁶ Nippon Biseibutsu Gakkai Zasshi, 1918, 6, p. 617.

⁷ Nippon Eiseibutusu Gakkai Zasshi, 1920, 12, p. 235.

injection of streptococci into the peritoneal cavity a sufficient number of polymorphonuclear neutrophils had migrated into the cavity for experiments on phagocytosis; hence, in all my experiments, the peritoneal fluid was examined 3 hours after the injection of streptococci. I used M/8 solutions of various salts, diluting when necessary with normal salt solution, and a typically hemolytic streptococcus isolated from the frontal sinus and cultivated artificially for about 2 years. The preparations were stained with Wright's method.

A mixture consisting of 0.5 c.c. of M/8 solution of salt, 0.5 c.c. of normal salt solution and 0.5 c.c. of a 24-hour growth of this streptococcus in plain broth was injected into the peritoneal cavity of the mouse. After 3 hours peritoneal fluid was withdrawn, preparations were made and the number of leukocytes engaged in phagocytosis, as well as the average number of streptococci taken up by each phagocytic leukocyte, was determined. Two sets of experiments were made, one with a nonvirulent strain of the streptococcus, a strain that had not been passed through animals, and a virulent strain obtained by passage through 6 to 8 mice in succession. The results are given in table 1, and they show that the various salts used inhibited phagocytosis of streptococci in the peritoneal cavity of the mouse. It is also shown that there is much less phagocytosis in the case of the virulent as compared with nonvirulent strain, which is in full accord with results of previous observation, notably those by Denys and Marschand, Neufeld and Hune, Hektoen⁸ and others.

Further experiments, the results of which are given in tables 2 and 3, were made in the same way with higher dilution of the same salts.

Observations were also made on the phagocytosis of streptococci *in vitro* in mixtures of normal rabbit serum, normal guinea-pig leukocytes and different salt solutions, equal quantities. These mixtures were incubated at 37 C. for one hour when smears were made (table 3), the results showing that higher dilutions of the salts used have no stimulating effect on phagocytosis, there being simply a diminution of the inhibitory power that lower dilutions exercise.

In the next experiment, guinea-pigs were injected subcutaneously with 3 c.c. of salt solution once a day for 5 days. After the last injection a suspension of avirulent streptococci was injected into the peritoneal cavity, preparations of the peritoneal fluid made 3 hours later and the degree of phagocytosis determined as before (table 4).

TABLE 1

INFLUENCE OF M/8 SALT SOLUTIONS ON PHAGOCYTOSIS IN THE PERITONEAL CAVITY OF THE MOUSE

Salts	Nonvirulent Streptococci		Virulent Streptococci	
	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes
NaCl.....	6.24	78	0.62	24
MgSO ₄	1.428	51	0.24	16
MgCl ₂	1.081	47	0.25	15
SrCl ₂	0.975	39	0.22	13
Na ₂ CO ₃	0.990	45	0.20	12
Na ₃ C ₆ H ₅ O ₇	0.840	42	0.22	15
CaCl ₂	0.916	39	0.21	14
KJ.....	2.480	62	0.32	17
KBr.....	3.450	69	0.35	18

TABLE 2

INFLUENCE OF M/100 AND M/300 SALT SOLUTIONS ON PHAGOCYTOSIS IN THE PERITONEAL CAVITY OF THE MOUSE

Salts	M/100 Solutions		M/300 Solutions	
	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes
NaCl.....	12.5	73	14.3	86
MgSO ₄	9.2	60	13.8	85
MgCl ₂	8.8	60	12.0	83
SrCl ₂	10.5	65	12.8	86
CaCl ₂	10.7	63	14.5	83
Na ₂ CO ₃	9.5	55	12.5	81
Na ₃ C ₆ H ₅ O ₇	9.1	62	13.0	78
K ₄ Fe(CN) ₆	7.3	52	10.0	75
KJ.....	10.7	69	12.2	75
KBr.....	12.0	70	12.0	79

TABLE 3

INFLUENCE OF DILUTE SALT SOLUTIONS ON PHAGOCYTOSIS IN VITRO

Salts	M/50 Solutions		M/200 Solutions	
	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes
NaCl.....	6.4	71	7.8	70
MgSO ₄	5.5	65	6.5	60
MgCl ₂	5.8	57	6.5	62
SrCl ₂	6.1	69	6.3	60
CaCl ₂	4.5	55	6.7	62
Na ₂ CO ₃	6.0	64	6.0	55
Na ₃ C ₆ H ₅ O ₇	5.5	64	6.0	60
K ₄ Fe(CN) ₆	3.5	43	6.1	58
KJ.....	5.8	64	6.8	67
No serum.....	1.3	19	1.3	15

⁸ Jour. Am. Med. Assn., 1906, 46, p. 1407.

TABLE 4

THE INFLUENCE OF INJECTIONS OF SALT SOLUTIONS ON PHAGOCYTOSIS IN THE PERITONEAL CAVITY OF THE GUINEA-PIG

Guinea-Pigs	Salts	M/8 Solutions		M/200 Solutions	
		Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes	Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes
Nonvirulent Streptococci					
1	NaCl.....	6.2	67	17.4	72
2	MgSO ₄	4.8	52	16.5	66
3	Na ₂ CO ₃	4.0	50	16.1	67
4	Na ₃ C ₆ H ₅ O ₇	3.9	48	15.8	69
5	CaCl ₂	4.0	46	17.7	70
Virulent Streptococci					
1	NaCl.....	12.1	77	19	83
2	MgSO ₄	5.5	37	17	78
3	Na ₂ CO ₃	5.1	40	17	80
4	Na ₃ C ₆ H ₅ O ₇	5.5	39	18	81
5	CaCl ₂	4.9	37	19	78

TABLE 5

THE RELATION OF ALKALINITY OF BLOOD AND PHAGOCYTOSIS OF STREPTOCOCCI IN THE PERITONEAL CAVITY

Guinea-Pigs	Salts M/8 Solution	Alkalinity of Blood		Average Number of Cocci in Each Leukocyte	Percentage of Phagocytic Leukocytes
		Before Injection of Salt	After Injection of Salt		
Normal guinea-pigs injected with nonvirulent streptococci	1 NaCl	3	3	17.6	74
	2 MgSO ₄	3	2	11.3	56
	3 Na ₂ CO ₃	3	2	8.3	43
	4 Na ₃ C ₆ H ₅ O ₇	3	2	8.0	38
	5 CaCl ₂	3	2	8.1	40
Immunized guinea-pigs injected with virulent streptococci	1 NaCl	2	2	15.7	63
	2 MgSO ₄	3	2	9.6	39
	3 Na ₂ CO ₃	2	1	7.5	34
	4 Na ₃ C ₆ H ₅ O ₇	3	2	8.0	38
	5 CaCl ₂	2	1	8.1	33

Guinea-pigs were immunized against the streptococcus by 4 intra-peritoneal injections given every 5 days, consisting of 2 to 6 c c of a 24-hour culture of streptococci in ascites broth; the culture had been heated to 60 C. for one hour. On the fourth day after the last injection, 3 c c of salt solution were injected subcutaneously; this was repeated for 5 days (table 5), the results indicating that the phagocytic power of the peritoneal fluid may be decreased by the repeated injection of M/8 solutions of the salts used.

LANDOIS' METHOD

Solution A = Tartaric acid, 7.5 gm., dissolved in 1 liter of water.

Solution B = Saturated solution of sodium sulphate.

Solution A	10.0 cc	+	Solution B	100.0 cc	=	0.036% NaOH
Solution A	20.0 cc	+	Solution B	90.0 cc	=	0.072% NaOH
Solution A	30.0 cc	+	Solution B	80.0 cc	=	0.108% NaOH
Solution A	40.0 cc	+	Solution B	70.0 cc	=	0.144% NaOH
Solution A	50.0 cc	+	Solution B	60.0 cc	=	0.180% NaOH
Solution A	60.0 cc	+	Solution B	50.0 cc	=	0.216% NaOH
Solution A	70.0 cc	+	Solution B	40.0 cc	=	0.252% NaOH
Solution A	80.0 cc	+	Solution B	30.0 cc	=	0.288% NaOH
Solution A	90.0 cc	+	Solution B	20.0 cc	=	0.324% NaOH
Solution A	100.0 cc	+	Solution B	10.0 cc	=	0.360% NaOH

Levy⁹ ascribes the increased susceptibility of diabetes to staphylococcus infection and tuberculosis to decreased alkalescence of the blood. A diminution of opsonin in the serum of diabetics was observed by DaCosta and Beardsley.¹⁰ Other investigators have also noted that increased alkalescence of the blood may be associated with a decrease in resistance to infection.¹¹ I, therefore, made some observations on the influence of certain salts on the alkalescence of the blood and phagocytic power. To determine the degree of alkalescence I used the method of Landois, the solution of the reagent being drawn up into a capillary pipet and then mixed with an equal quantity of fresh blood from the ear vein; the mixture was then tested with litmus paper. This method of testing must be continued until the particular reagent solution is found that, when mixed with blood, is neutral to litmus, the corresponding amount of alkali giving the degree of alkalinity of the blood. I made observations of this kind on normal guinea-pigs, immunized guinea-pigs and normal rabbits, and injected salt solution subcutaneously once a day for 5 days. The alkalinity of the blood was tested before the first injection and the day after the last injection, when streptococci were injected into the abdominal cavity, smears being made 3 hours later. The results are given in tables 5 and 6. The immunized guinea-pigs received 4 intraperitoneal injections, one every 5 days, consisting of from 2 to 6 cc of a heated 24-hour ascites broth culture of streptococci. The first injection of salt solution was given on the fourth day after the last injection of pure streptococci, the alkalinity of the blood being determined just before and the day after the injection of salt solution. In the

⁹ Arch. f. Exper. Path. and Pharmacol., 1899, 42, p. 149.

¹⁰ Am. Jour. Med. Sc., 1908, 136, p. 361.

¹¹ Ceni, Handb. d. Path. Mikroorg., Kolle and Wassermann, 1912, 1, p. 1001; Innocent and Zagari, *ibid.*, p. 1004; Onodera, Nippon Naikagakukai Zasshi, 1920, 7, p. 626.

case of the rabbits 5 c c of salt solution were injected subcutaneously every day for 10 days. The results indicate that there may be some diminution in the alkalinity of the blood, as determined by the method used after the injection of various salts, and that at the same time there is a reduction in phagocytic power. No difference was observed in the results with normal and immunized guinea-pigs.

TABLE 6

RELATION OF ALKALINITY OF BLOOD TO PHAGOCYTIC ACTIVITY OF RABBITS INJECTED WITH SALT SOLUTIONS AND NONVIRULENT STREPTOCOCCI

Rabbits	Salts M/8 Solution	Alkalinity of Blood		Average Number of Cocci in Each Leuko- cyte in Mixtures Made with Serum of Blood		Average Number of Cocci in Each Leukocyte in Peri- toneal Fluid	Percentage of Phagocytic Leukocytes in Perito- neal Fluid
		Before Injec- tion of Salt	After Injec- tion of Salt	Before Injection of Salt	After Injection of Salt		
1	NaCl	3	3	13.5	12.0	8.9	75
2	MgSO ₄	3	2	14.5	7.0	3.6	35
3	Na ₂ CO ₃	4	2	14.0	5.0	2.55	31
4	Na ₃ C ₆ H ₅ O ₇	4	2	16.0	5.0	2.6	29
5	CaCl ₂	4	2	15.0	5.2	2.7	30

TABLE 7

INFLUENCE OF SALT SOLUTION ON CHEMOTAXIS IN THE PERITONEAL CAVITY OF THE MOUSE

Salts	M/S Solution	M/300 Solution	M/600 Solution
MgSO ₄	16,050	19,100	19,700
MgCl ₂	13,000	17,700	17,200
Na ₂ CO ₃	16,300	17,900	18,500
Na ₃ C ₆ H ₅ O ₇	15,100	18,400	22,800
CaCl ₂	13,000	20,010	20,070
KJ.....	13,500	18,700	23,100
NaCl.....	20,500		

The figures give the number of leukocytes in 1 c.mm.

Hamburger¹² observed that a dilute solution of calcium chloride is strongly chemotactic. I made some experiments using the following method: Equal parts of salt solution and plain broth were injected into the peritoneal cavity of mice and 3 hours later the number of leukocytes in the fluid was determined (Thoma-Zeiss), the results showing that M/S solutions of the salts used attracted less leukocytes than normal salt solution, this effect not obtaining with higher dilutions of the salts.

I also studied the influence of some salts on the virulence and other qualities of streptococci. For this purpose 0.5 c c of salt

¹² Biochem. Ztschr., 1910, 26, p. 66.

solution were mixed with a quantity of streptococcus suspension just before intraperitoneal injection into mice. The salts used in this set of experiments were NaCl, MgSO_4 , Na_2CO_3 and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, and the original streptococcus strain that had been isolated from the frontal sinus. In the case of each salt-streptococcus mixture (24-hour ascites broth culture), the minimal fatal dose was determined. After the death of the mouse, cultures were made from the heart blood, and after 24 hours ascites broth was inoculated from a single colony and incubated for 24 hours, when new mixtures were made with the salt solution and a new series of mice received injections. After from 16 to 18 passages made in the manner outlined, the minimal fatal dose for mice in 72 hours of the cultures obtained from the last passage was determined with the following results:

NaCl-streptococcus mixture	0.007 c c
MgSO_4	0.003 c c
Na_2CO_3	0.004 c c
$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	0.002 c c

It appears that the NaCl strain was weakest and the $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ strain was the strongest in virulence.

Suspensions of these salt strains of streptococci in 0.9% NaCl solution were centrifugated and shaken with small glass balls to break up the chain;³ agglutination tests were made with an antistreptococcus serum with the result that the $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ strain agglutinated at a dilution of 1:160 only, whereas the other strains agglutinated at a dilution of 1:640. Phagocytosis experiments with immune serum gave similar results. For streptolysin tests streptococci were inoculated from blood agar into 10 c c of equal parts of plain broth and ascites fluid (heated at 56 C. for 30 minutes), incubated for 18 hours and then centrifugated, the supernatant fluid being used for the test. In all cases the sterility of the medium was tested carefully before inoculation. Washed blood corpuscles from various animals were used in the test; suspensions of corpuscles and the centrifugate of the streptococcus cultures were mixed and incubated for 2 hours, the results being read after 24 hours in the icebox. It was found that even though the virulence of the streptococci had increased by passage through mice together with different salt solutions, the production of lysin showed a decrease (table 8). The fermentative power of the different salt strains of streptococci was also tested, 1% sugar broth (all the usual

sugars) being inoculated directly from blood agar; 90 c c of distilled water was added to 10 c c of the centrifugated broth culture 24 hours old and titrated with N/10 at the boiling point, phenolphthalein being indicated. While there was a slight variation in the quantity of acid produced by the different strains, no marked difference in the power of sugar fermentation was detected.

SUMMARY

Phagocytosis of streptococci in the peritoneal cavity of the mouse is diminished by M/8 solutions of certain salts (MgSO_4 , Mg Cl_2 , Sr-Cl_2 , Na_2CO_3 , $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, and CaCl_2). Ki and KBr inhibit in lesser degree.

TABLE 8
PRODUCTION OF LYSIN BY DIFFERENT SALT STRAINS OF HEMOLYTIC STREPTOCOCCUS

Salt Strains of Streptococcus	Corpuscles			
	Mouse	Rabbits	Guinea-Pig	Sheep
Original culture.....	0.0015	0.003	0.0015	0.006
NaCl strain.....	0.003	0.006	0.006	0.025
MgSO_4 strain.....	0.006	0.012	0.012	0.05
Na_2CO_3 strain.....	0.006	0.012	0.006	0.05
$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ strain.....	0.012	0.025	0.025	0.75

Higher dilutions of these salts seem to have no stimulating effect on phagocytosis in vitro or in vivo. As the dilution increases the inhibitory power is lessened. Repeated injections of some of the salt solutions used appear to reduce phagocytosis in the peritoneal cavity, but no stimulating effect was obtained by subcutaneous injection of higher dilutions of the salts in question. The results were the same in normal and immunized guinea-pigs.

Each mixture contained in 1 c c of a 5% suspension of corpuscles and 1 c c of streptococcus filtrate and salt solutions, the total quantity being always 2 c c. In the table the figures indicate the smallest quantity of streptococcus filtrates that produce definite lysis under the circumstances outlined.

Repeated subcutaneous injections of certain salts reduced the alkalinity of the blood and also the phagocytic power of the peritoneal exudate.

Fewer leukocytes appear in the peritoneal cavity 3 hours after the injection of M/8 solutions of MgSO_4 , etc., than after the injection of normal salt solution.

When solutions of certain salts are injected together with streptococcus suspension in mice and the procedure repeated through many successive passages, some salts may have a greater influence on the character of the streptococcus than others. Thus $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$ seemed to favor the development of virulence, to reduce agglutinability and phagocytibility as well as the power to elaborate lysin, but it had no special effect on the power to ferment sugars.

A SPONTANEOUS EPIDEMIC AMONG LABORATORY
RABBITS CAUSED BY A PARATYPHOID
B. BACILLUS RELATED TO
THE RODENT GROUP

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Spontaneous bacterial infections among laboratory or breeding rabbits are not uncommon. Irrespective of the confusing nomenclature,¹ which may at first suggest a variety of microbial causes, the etiology of such epidemics is in our experience usually confined to *B. cuniculisepticus* and *B. bronchisepticus*. Recently our attention was called to the occurrence of true paratyphoid *B.* infections.

According to the available information, epidemics among rabbits caused by this group of organisms are rare. Uhlenhuth and Hübner² mention, in their summary on the paratyphoid group, that Holst and Hottinger determined bacteria of this group to be the cause of spontaneous epizootics among rabbits. Also Pfeiler³ expresses the same idea in a short sentence in one of his recent reviews. A perusal of the articles of Holst and Hottinger referred to did not inform us of the facts on which these statements are based. In his complete review Loele⁴ does not mention Holst and Hottinger. A definite description of paratyphoid fever in rabbits as a disease entity is therefore not available although Morgan⁵ obtained three cultures of an organism like paratyphoid A from rabbits' feces, an observation that we were able to confirm. In 1914 Ferry⁶ made an intensive study of the causative organisms found in *B. bronchisepticus* epidemics among laboratory animals. In a few cases he was able to isolate an organism of the paratyphoid *B. enteritidis* group; he considered these bacteria to be secondary invaders superimposed on an infection with *B. bronchisepticus* or *B. cuniculisepticus*. Krumwiede, Valentine and Kohn⁷ have recently published an article in which they show that paratyphoid strains isolated from guinea-pigs, mice, rabbits and cats differ antigenically based on specific absorption tests from human paratyphoid B. or *B. schottmülleri*-strains. A distinct type or group, the so-called "rodent paratyphoid," is according to their views encountered in spontaneous infections of laboratory animals, especially rodents.

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¹ Hutyra and Marek: *Spezielle Pathologie & Therapie d. Haustiere*, 1913, 1, p. 104; Ferry and Hoskins: *Jour. Lab. & Clin. Med.*, 1920, 5, p. 311.

² Kolle and Wassermann: *Handb. d. pathog. Mikroorganis.*, 1913, 3, p. 1102.

³ Friedberger and Pfeiffer: *Lehrbuch d. Mikrobiologie*, 1919, 2, p. 917.

⁴ *Ergebn. d. allg. Pathol. u. Pathol. Anatomie*, 1915, 18, p. 628.

⁵ *Brit. Med. Jour.*, 1905, 1, p. 1257.

⁶ *Jour. Path. & Bacteriol.*, 1914, 18, p. 445.

⁷ *Jour. Med. Res.*, 1919, 34, p. 449.

In this connection the report of MacConkey⁸ on some cases of food poisoning should also be recalled. This worker isolated a paratyphoid B. bacillus from the hind limb of a rabbit, the only part of a meal which had apparently caused three cases of food poisoning and the death of a 6 months old baby. This organism was identical with the nonlaetose fermenting bacteria obtained from the intestines of the baby. Certain facts presented by MacConkey make it certain that when purchased the rabbit was fit for human consumption and contamination must have taken place at the consumers' house. The possible importance of the observation of MacConkey will be considered separately more in detail.

In the light of this information it appeared profitable to investigate more carefully the spontaneous epidemic that occurred in our animal house. This study was particularly valuable in furnishing a rabbit-pathogenic paratyphoid bacillus and a number of data, which assisted us materially in the analysis of the factors of immunity operative in experimental typhoid carriers of this species. We shall have occasion to refer to the organisms isolated from our epidemic in other papers and consider for the present only the symptoms, the necropsy and microscopic findings, the organism isolated, its serologic classification in the paratyphoid group and its pathogenicity for various animals.

HISTORY OF OUR EPIDEMIC

Nov. 26, 1918, two lots of rabbits were received in the laboratory. One set came from a reliable dealer; his rabbits had not, during the past two years, shown signs of coccidiosis or "snuffles." The second lot was sent out by a breeder unknown to us, who had been recommended as always having been successful in raising rabbits. When these rabbits were unpacked, one female was found to have diarrhea and signs of a previous abortion. This animal was immediately isolated but had already been in intimate contact with 12 others of the same lot. This visibly diseased rabbit died Nov. 8 and was only superficially examined. No attention was paid to lung lesions and no cultures were taken. Death was attributed to a polybacterial puerperal septicemia, not uncommonly observed in rabbits closely packed and shipped in unsuitable boxes.

Dec. 8 and 9 two rabbits, that had been in contact with this animal, succumbed. Both animals had diarrhea and extensive bilateral croupous pneumonia. Cultures of paratyphoid B. bacilli were obtained from the lung tissues of both rabbits. The anatomic lesions did not suggest additional cultures. However, the lung lesions differed in many respects from those ordinarily encountered in our laboratory and indicated, even before we had obtained and studied the cultures, that we were not dealing with one of the common, intercurrent infections. A careful quarantine and a daily inspection of the stock animals was immediately instituted.

A fourth rabbit, rabbit 1371, was found to be sick on Dec. 11. It refused all food, had a purulent nasal discharge and diarrhea, and its coat was shaggy and rough. This large animal, weighing 3150 gm., was chloroformed. The anatomic lesions were typical of those commonly found in experimentally produced paratyphoid infections. Cultures were obtained on direct plating from all the organs, except the bile. The heart blood contained 720 colonies per c.c.

Dec. 13 a fifth rabbit, 1372, was found dead. This animal had not been in contact with the previously mentioned rabbits and had been placed in a single

⁸ Jour. Hyg., 1906, 6, p. 570.

isolation cage of the infectious disease room the day before. It had anatomic lesions similar to 1371. The intestinal sloughs were particularly well marked in the appendix region. Cultures were also obtained from all of the organs. Epidemiologically this animal was probably infected by the careless use of cleaning utensils employed in the removal of the manure. Previous to Dec. 8, when we were not as yet fully informed as to the nature of the disease, the caretaker used the same scraper on the cage holding rabbit 1372 that he had used in cleaning the section in which rabbits 2 and 3 had succumbed. It was quite obvious that only rigorous measures would interrupt the chain of continuous contact infections. Thorough disinfection of all the suspected cages with lysol and lime, sterilization of the cleaning utensils, food and drinking cups accomplished the desired result. Further cases have not occurred.

SYMPTOMS AND LESIONS

Only two spontaneously infected and several artificially infected (feeding) rabbits were available for a study of the clinical symptoms. After an incubation period of from 2 to 3 days the animals invariably had fever, the temperature being above 40 C., reaching 41.3 C. on one occasion. The hair was rough and shed more readily. In two animals a seropurulent nasal discharge was noticed. Usually they refused to eat, but drank much water. The total loss of weight varied with the individual case but was noted in every instance. There was marked pallor of the mucous membranes due to a distinct drop in the hemoglobin from 90 to 52%. A few animals showed a slight hyperleukocytosis. Every animal developed severe diarrhea preceding death by several days. This symptom was frequently accompanied by a paresis of the posterior extremities and involuntary passage of urine. There was a quickened pulse, labored breathing and general prostration, a drop in temperature and convulsions signaled the approaching termination in from 4 to 8 days after the onset of the symptoms.

The gross lesions were: Emaciation was always more or less marked. The left or right anterior lobe of the lung of the spontaneously infected animals was as a rule covered with a thin layer of fibrin, the lung parenchyma was consolidated in lobar distribution or showed an infarct-like area surrounded by patchy pneumonia. The anterior portion of the left or right middle lobe showed red and gray hepatization. Also the right anterior and heart lobe were similarly affected and the pleural covering of the pericardial sac frequently showed a fine network of fibrin. The bronchi contained a small amount of grayish purulent exudate. The heart muscle was flabby and decidedly fatty. The blood was thin and sometimes of a brownish tinge. The spleen, which was dark brown, engorged, pulpy, and was enlarged, for example, to $8 \times 1.7 \times 1.5$ cm., weighing 6 to 14 gm. (The normal weight averages between 0.45 - 1.1 gm.) Both kidneys were enlarged, yellowish brown in color, petechiated and showed extensive parenchymatous degeneration. The mesenteric lymph nodes were enlarged, soft and juicy, and contained numerous small or large hemorrhages. Not infrequently the hepatic, peri-aortic and iliac lymph nodes were enlarged and distinctly hyperemic. The liver was increased in size and either dark or pale in color, friable and dotted with small necrotic foci of varying sizes. Along the margins large subcapsular brownish-yellow patches were noted. The gallbladder was distended by a dark, olive green, viscid bile; the wall was thin and soft; no changes were visible on the mucous membrane. In the rabbits infected by intravenous injection the gallbladder was leathery, necrotic and the bile thin or thick and purulent.

The bone marrow of the femur was a light yellowish-brown, soft or deep red and showed a few areas suggestive of necrosis. The stomach externally and internally seemed normal. Stringy, bile-tinged mucus filled the duodenum, the mucous membrane was slightly swollen and was sometimes petechiated. All

the Peyer's patches of the jejunum were swollen, deep red, ulcerated or covered by brownish-red scablike sloughs. The contents were greenish and frothy. Sacculus rotundatus was considerably thickened; the lymphatic structure studded with numerous deep hemorrhages and small pinhead-like abscesses. The mucous membrane was covered by a glassy, slightly blood-tinged slime, and in two spontaneously infected animals consisted of extensive cauliflower-like sloughs. In one rabbit the appendix mucous membrane was banded by broad hemorrhagic areas 0.5 cm. in width or dotted irregularly by diphtheric hemorrhagic round or irregular sloughs. A glassy blood-tinged mucus covered the completely or partially necrotized mucous membrane. The extent of these intestinal lesions varied considerably. The artificially fed rabbits (bile method of Besredka) presented a strikingly rigid cecal or colonic wall, together with a diffuse diphtheric necrosis of the mucous membrane in contrast to the scattered ulcerations observed in the spontaneously infected animals.

Rabbits immunized with living organisms or those that had recovered from an acute infection invariably showed pinhead-like whitish or yellowish abscesses in the lymphoid tissues of the sacculus rotundatus or appendix; such findings have been recorded by Theobald Smith⁹ in rabbits infected with *B. suis*. These abscesses were either sterile or contained streptococci. The uterine horns of two rabbits were enlarged, contained partially dissolved putrid fetuses and an endometrium covered with yellowish necrotic areas. In one male rabbit the epididymis were edematous, hemorrhagic and showed on section numerous areas of necrosis. An orchitis was not present. Sexotropic properties apparently characterized the organism of this epidemic.

The histologic examination of some of the organs revealed changes not commonly encountered in paratyphoid infections of laboratory animals. An endarteritis and endophlebitis were noted in every tissue, not only in the spontaneously but also in some of the experimentally infected animals. Again the characteristic hyperplastic reaction regularly found in human typhoid fever was entirely absent or indicated only by a few scattered macrophages and phagocytes. These observations were unfortunately recorded only when our organisms had already lost their original virulence, and additional infection experiments were mostly unsuccessful or resulted in morbid lesions different from those seen in the spontaneous cases. A careful histologic study of paratyphoid infections should be undertaken in order to elucidate the pathogenesis of this group of organisms. Such a study is particularly desirable in the light of the recent reports of Huebschmann,¹⁰ Herxheimer,¹¹ Dawson and Whittington,¹² and others who noted not only bacteriologic but also fundamental anatomic differences between paratyphoid B. and true typhoid infections in man. Our incomplete data are presented in order to encourage such research on spontaneously infected animals when the occasion arises, and they should not be considered an exhaustive inquiry into this phase of the infection.

The pneumonic changes described apparently developed in three of the spontaneously infected animals as a bronchopneumonia with subsequent lobar red and gray hepatization. This associated type of pneumonia developed as a result of pronounced inflammatory processes in a large number of the inter-alveolar arteries and veins. The lumen of these vessels was filled with nests of fragmented and normal leukocytes of varying origin. The endothelium was loosened from the intima and the media and with the adventitia were edematous and invaded by leukocytes. In some vessels fibrin deposits and bacterial plugs

⁹ U. S. Department of Agriculture, Bureau Animal Industry, Bulletin No. 6, 1894, p. 25

¹⁰ Beitr. z. path. Anat. u. z. allg. Path., 1913, 56, p. 514.

¹¹ Berl. klin. Wchnschr., 1916, 53, p. 648

¹² Quart. Jour. Med., 1916, 9, p. 98.



Fig. 1.—Anatomic lesions in rabbit 1371: (1) transverse section of left anterior lobe; (2) kidney; (3) appendix vermiformis opened; (4) mesenteric lymph nodes; (5) incised Peyer's patch; (6) sacculus rotundatus opened, hemorrhagic slough on mucous membrane.

were recognized. Surrounding these inflamed vessels were alveoli containing coagulated albuminous material, fibrin, red cells or leukocytes or even infarcted-like areas. Certain complexes of lobules showed incipient or advanced stages of necrosis, and these areas fused with the bronchopneumonic patches of the lung tissue. The extensive involvement of the terminal bronchi, which were filled with leukocytic plugs extending into the neighboring air cells, suggests that these animals suffered in all probability previous to the paratyphoid infection from the common bronchitis due to *B. cuniculisepticus*. It should, however, be remembered in this connection that Dawson and Whittington, Herxheimer and particularly Klein and Torrey¹³ consider bronchitis and bronchopneumonia as important anatomic lesions in human paratyphoid *B.* infections. Artificially infected rabbits, however, failed to show similar pulmonary changes, which supports the contention of a mixed infection.

The liver sinusoids of all the animals spontaneously or artificially diseased were moderately engorged with blood. The peripheral liver cells contained large fat droplets. Numerous areas consisting of necrosed liver cells and accumulated leukocytes in various stages of fragmentation or typical toxic "pseudo tubercles" were common. In some instances these focal necroses were around the central vein, in others they involved several lobules. Invariably the portal veins or spaces contained thrombotic material and exhibited definite zones of infarction. We have the impression that the necrosis is the end result of a toxin action and not the outcome of embolic blocking of the sinusoids by leukocytes or even splenic cells. Our observations are in this connection quite in accord with those reported by Gruber¹⁴ and Wagner and Emmerich.¹⁵ Bacterial clumps were occasionally seen in the areas of focal necroses. Typical "lymphomas," consisting of macrophages, were not observed. The gallbladder wall was only involved in the intravenously injected animals. The importance of this fact will be discussed below.

The follicles or germ centers of the spleen were usually enlarged and showed a distinct toxic hyaline degeneration ("eosinophilic hyperplasia") or were completely submerged in the pulp changes. The pulp was engorged with red cells or pigment debris partially inclosed in cells. Numerous necroses, thrombi or fibrin, phagocytic and desquamated pulp cells and nuclear fragments in the splenic sinuses formed a complicated picture. Macrophages were not found, but nests of leukocytes well preserved or partially disintegrated were not uncommon. The cortical sinuses of the mesenteric and portal lymph nodes were slightly distended by leukocytes and hyaline-like debris; the central sinuses, particularly near the hilum, were packed with red cells and large red cell carrying phagocytes and polymorphonuclear leukocytes. No hyperplasia of the follicles or the medullary cords were noted in the large number of nodes examined for this purpose. The spontaneously infected rabbits showed, however, distinct endarteritic changes in several small arteries and patches of more or less marked necrosis in the follicles.

In the jejunum dilatation of the blood vessels and catarrhal hypersecretion of the mucous glands were the most striking changes recorded. The Peyer's patches showed either extensive hemorrhages or complete necrosis of the mucous membrane covering the lymphatic tissue. These changes were accompanied by leukocytic infiltration, at times forming a distinct zone of demarcation. Invariably the submucosal blood or lymph vessels were plugged with leukocytes or thrombotic material. The necrotizing diphtheria inflammatory processes of the sacculus rotundatus, appendix and ileum were confined to the

¹³ Am. Jour. Med. Sci., 1920, 159, p. 546.

¹⁴ Centralbl. f. Bakteriol., O., I, 1916, 79, p. 1.

¹⁵ Ibid., 1916, 77, p. 301.

intestinal wall provided with lymphatic tissue. In these places a confluent mass of necrosis, together with an extensive hemorrhage and little or no fibrin, covered an area of cellular degeneration and disintegration extending to the oedematous muscularis.

The diphtheric changes in the colon of the rabbits infected by first feeding bile and following with the ingestion of large amounts of paratyphoid bacilli resemble those of epidemic dysentery. A phlegmonous infiltration of the entire wall of the mucous membrane was covered by a layer of fibrin and necrotic tissue.

The mucous membrane of the appendix in one rabbit was in its entire distribution necrotic and appeared as a faintly stained mass resting on a circular zone of demarcation just above the lymphatic tissue layer. In some of the immune or recovered animals the follicles of this portion of the appendix contained large areas of necrosis and leukocytic aggregations forming a micro abscess. There were changes in the regional capillaries, but not as marked as those described in the lung and the mesenteric lymph nodes. The lesions are unquestionably different from those reported in human typhoid. They develop in the lymphatic tissues of the intestinal canal but not on the basis of a hyperplastic reaction. Hemorrhages and necroses, apparently the result of vascular injury, predominate and in this respect the anatomic process of "rabbit typhoid" finds an analogy in human paratyphoid or in hog cholera. As far as the appendix is concerned, the lesions remind the observer strongly of those seen in acute human appendicitis; certain stages are strikingly similar to those described by McMeans¹⁶ in his able article on experimental appendicitis.

Some sections of the bone marrow exhibited hemorrhages, leukocytic accumulations and in one instance small scattered areas of necrosis. The myocardium of one rabbit (1415) showed extensive fatty degeneration of the muscle fibers; in the majority of animals indications of a beginning interstitial myocarditis were visible. The blood vessels of the epicardium and myocardium were dilated and frequently possessed nests of leukocytes.

Hyaline thrombi in the glomerular capillaries, hemorrhages, irregular degeneration of the tubular lining, perivascular edema and albuminous tufts were noted in the kidneys. The suprarenals were normal. In one male rabbit the spermatic vein contained thrombotic material and leukocytic debris with adjacent necrosis of a portion of the epididymis. The uterus of two animals contained necrotic embryonic tissue and the endometrium possessed all the signs of a marked inflammation with necrosis. Clinically, as well as anatomically, this spontaneous paratyphoid infection in rabbits differed in no respect from the disease constantly observed and carefully described for this species inoculated with various representatives of the paratyphoid-enteritidis group, by Smith and Moore,¹⁷ Karlinski,¹⁸ Raccuglia,¹⁹ Ordway, Kellert and Husted,²⁰ Okubo,²¹ and others. The descriptions given by these writers cover our observations in every detail as far as the gross morbid lesions are concerned, but we are unable to find the endothelial hyperplasia and the typhoid-like microscopic changes mentioned repeatedly by Ordway and his associates. Vascular changes and necroses predominated in the tissues of our animals. It is not unlikely that the high virulence of the organism and the comparatively short duration

¹⁶ Arch. Int. Med., 1917, 19, p. 709.

¹⁷ U. S. Dept. of Agriculture, Bureau of Animal Industry, Bull. No. 6, 1894, p. 24.

¹⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 373.

¹⁹ Arb. a. d. Gebiete d. path. Anat. u. Bakteriell., 1891-1892, 1, p. 223.

²⁰ Jour. Med. Research, 1913, 28, p. 41.

²¹ Am. Jour. Dis. Children, 1918, 26, p. 376.

of the disease in our rabbits in comparison to the prolonged course of the infection in the observations of Ordway are in part responsible for the differences. However, in the light of recent knowledge on the morbid anatomy of paratyphoid fever in man, the microscopic findings have more than academic interest and urgently deserve additional investigations on suitable material. Such a study appears to us particularly profitable, when we appreciate that the information concerning the factors of pathogenesis, toxigenesis and immunity of the typhoid-paratyphoid group must indeed be considered meager.

THE ORGANISM

Impressions of tissue sections or fragments and direct quantitative plating of the secretions of the spontaneously infected rabbits on brilliant green-cosine-agar or peptic digest endo-agar gave a pure growth of abundant colonies of a gram-negative, motile bacillus. The heart blood of one rabbit (1371) produced 720 colonies per c.c. From the intestinal content, the wall of the jejunum, ileum, sacculus rotundatus and cecum, the liver, the spleen, the lung, etc., a practically pure and profuse growth of the same organism was obtained. The bile did not contain viable bacteria on direct plating of 1 c.c.; on enrichment in broth paratyphoid-like organisms could be demonstrated.

The isolated organisms fermented the following carbohydrates, with the formation of gas, glucose, maltose, levulose, mannite, galactose, dulcitol, mannose, arabinose, sorbitol, rhamnose, and xylose, but had no effect on lactose, saccharose, dextrin, inulin, salicin, raffinose, dextrin and adonite. The fermentation of inositol was slow; acidification was noted only after 48-60 hours' incubation. In lead acetate agar, there was a browning of the medium at the end of 24 hours, and glucose serum water, according to Krumwiede, was reduced. Neutral red glucose agar was split by gas production and reduced. In bromocresol-purple-milk, the reaction at the end of 24 hours was slightly acid, and remained so for 48-72 hours. After the fourth day the medium turned gradually alkaline and at the end of 20 days was slightly saponified. Gelatin was not liquefied in 30 days and no indol was produced in Difco or Witte's peptone solution. The organisms are highly resistant to brilliant green. The final acidity in glucose diacid phosphate-peptone solution was P_H 4.8-5.0 after 48 hours and P_H 5.0 in mannite after the same period of incubation. The biochemical reactions just mentioned place the organisms isolated with the *B. schottmülleri* type of the colon-typhoid group. The behavior in carbohydrate mediums followed the type III reaction described by Winslow, Kligler and Rothberg in 1917.

AGGLUTINATION

The strains isolated from the various tissues were readily agglutinated in the first generation by a polyvalent human paratyphoid B. (*B. schottmülleri*) serum in a dilution of 1:500 and were not influenced by a highly potent *B. enteritidis* serum in dilutions above 1:10. Subsequent serologic tests were therefore conducted with the representatives of the paratyphoid-suipestifer group. The technique employed in order to classify the rabbit organism was identical to the one recently described by one of us.²²

Specific antisera against paratyphoid B. strains of rodent, avian, porcine and human origin were obtained by subcutaneous injections of living organisms in progressive amounts varying from 0.01 c.c. to 0.2 c.c. of a 24-hour old broth culture at weekly intervals. This mode of immunization produces characteristic local necroses and abscesses.

²² Feusier and Meyer: Jour. Infect. Dis., 1920, 27, p. 185.

According to the chart the bacillus (lung strain 2 and the heart blood strain 1371 behave in an identical manner) is by direct agglutination a paratyphoid *B.* closely related to the rodent type of this large group. It has also been definitely proved that the strain does not belong to either the human, avian (true paratyphoid strains not *B. sanguinarum* or *B. pullorum*) or porcine paratyphosus "B" group. A rabbit antiserum prepared with our organism possessed group agglutinins for the guinea-pig-paratyphoids, for 2 avian and for 2 swine typhus strains. A rabbit and a guinea-pig serum produced with a guinea-pig-paratyphoid strain exhibited group affiliations to our bacillus and to a number of guinea-pig organisms isolated from different epidemics. The striking group agglutination reactions among the avian paratyphoids by these and other serums are noteworthy. A culture isolated by Dr. N. S. Ferry from a rabbit, and sent to us as a representative of the paratyphoids found by him in these animals, behaved more like a member of the avian than of the rodent group. The mutual cross agglutination among the guinea-pig and the avian paratyphoids is strongly suggestive of a close relationship. We are not in possession of the strains employed by Krumwiede, Valentine, and Kohn, and we can therefore not state conclusively that our organism belongs to their group.

Absorption tests failed to classify our organism further in the animal paratyphoid group. Antigenically the rabbit paratyphoid strains are members of the animal paratyphoid group, which can be readily distinguished by absorption tests from the true human *B. paratyphosus*, "B" or *B. schottmülleri* the causative organism of human paratyphoid fever. Further subgrouping of the animal paratyphoid into rodent, avian or porcine groups may be accomplished by direct agglutination, but a more conclusive classification is impossible by the customary absorption technic.

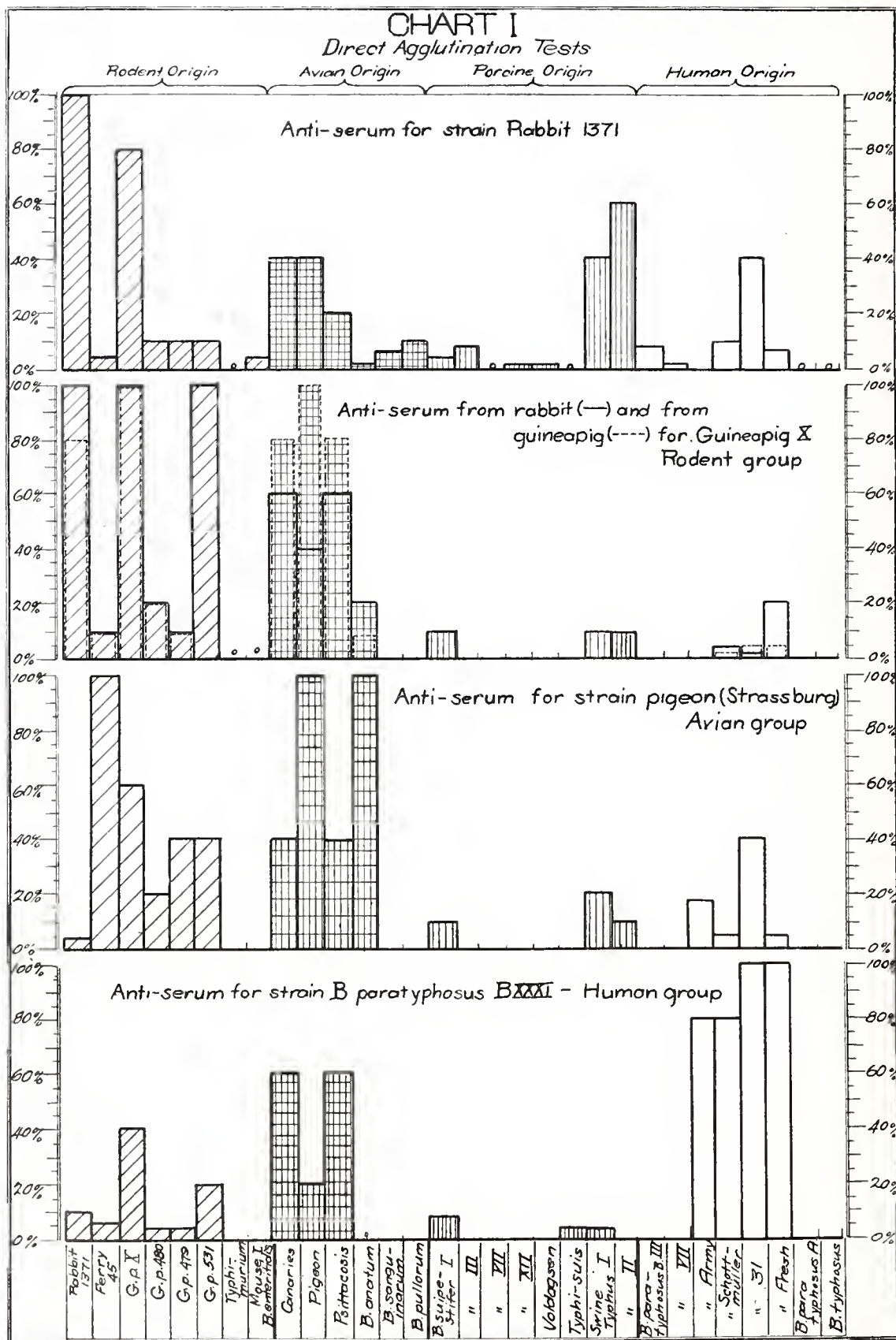
The hog cholera bacillus or *B. cholerae suis* represented in our collection by a number of strains designated as *B. suipestifer* and *B. voldagsen* and *B. typhi suis* do not, according to the tests of others and our own, belong to the rabbit group of animal paratyphoids, but are to be classed in a group by themselves. Some paratyphoid strains, of porcine origin, the so-called swine typhus culture I and II are related to our rabbit organisms. These observations support the recently formulated conclusion of Ten Broeck²³ relative to the existence of a distinct variety of paratyphoids common to animals and justify the acceptance of the terminology proposed by him. The organism could be designated as a member of the "*B. aertryckei*-group." Unquestionably further study in this large group of bacteria will, in our opinion, reveal additional subgroups with distinct host relationship.

PATHOGENICITY

The rapid progress of the epidemic obviously suggested a virulent strain of paratyphoid bacilli. As transmission from rabbit to rabbit in all probability resulted by way of the intestinal tract in form of a feeding infection, it was of interest to determine the approximate number of bacteria necessary to cause a fatal issue in these animals. It is generally stated and quite recently emphasized by Besredka²⁴ that the rabbit is nonsusceptible ("tout à fait invulnérable") against massive doses of paratyphoid or typhoid bacilli introduced into the digestive tract. This statement deserves some modification. Occasionally recently isolated, parasitic animal and human strains were encountered which can produce typical lesions and generalized infections when administered by

²³ Jour. Exper. Med., 1920, 32, p. 19.

²⁴ Bull. de l'Inst. Pasteur, 1920, 18 p. 123.



mouth. From a comparative standpoint it was also important to test our parasitic and saprophytized organism on other laboratory animals, particularly mice and guinea-pigs.

Inoculation Experiments.—Mice and guinea-pigs inoculated subcutaneously with 0.1-0.01 c.c. of a 24-hour broth culture succumbed in from 12 to 24 hours. The inoculated organism was found in the heart blood and in the organs. Even 15 months after isolation the strain retained approximately the same degree of virulence.

Subcutaneous inoculations of 0.1-0.01 c.c. of a 24-hour broth culture 2½ months after isolation produced in a large number of rabbits more or less extensive necroses and local abscesses. A rise in temperature for several days, loss of appetite, perhaps temporary diarrhea and an enlargement of the regional lymph nodes accompanied the local processes. Ten days after the inoculation the blood serum of such rabbits agglutinated the paratyphoid bacillus in dilutions from 1:400-1:800. Two additional injections produced local lesions only and usually immunized the animals completely as will be shown in another paper. Judging from the observations of Ten Broeck²⁵ with *B. suis*, our bacillus possesses in comparison a low virulence on subcutaneous application and our findings lend little support to the supposition that fleas or other insects could be considered as factors in the epidemic.

The intravenous inoculation, on the other hand, was fatal in comparatively small doses in from 2-10 days. The number of recently isolated cultures necessary to kill adult rabbits of different litters and age varied between 200,000 and 700,000 organisms in broth cultures of different compositions and reactions. The six month old strain was pathogenic in doses of 1-2 billion and after one year this number of bacteria was fatal only in young rabbits. Irrespective of the variation in virulence the clinical symptoms, following a distinct incubation time and the anatomic lesions of a septicemia, were practically the same. Of particular interest are the findings of a diphtheric or even necrotizing cholecystitis with positive cultural results in every nonimmunized rabbit which succumbed to the intravenous injection.

These experiments only demonstrate that the rodent paratyphoid bacillus possessed for laboratory animals an average virulence, which differed in no respect from the one ordinarily noted with recently isolated human or animal paratyphoid strains.

Feeding Experiments.—In the feeding experiments on coccidiosis-free rabbits the cultures were grown on agar slants and in peptic digest broth. Only cultures 24 hours old were used. The data obtained indicates that the ingestion of 2 billion recently isolated organisms produced the disease with characteristic lesions. When the strain was 6 months old the results became irregular with amounts as high as 8 billion, and when 12 months old even 1,600 billion were innocuous in a large series of animals. At the time of writing enormous doses (5,000 billion in milk) are necessary to produce an infection by feeding. Even 20-50 billion organisms are required to confirm the observations of Besredka²⁶ of feeding bile preceding the administration of organisms of the dysentery-paratyphoid group. The virulence of the organism has progressively degenerated and infected rabbits shedding these paratyphoid organisms can with impunity be mixed with susceptible ones. On the other hand, the epidemiologic data presented indicated a high original virulence which unquestionably

²⁵ Jour. Exper. Med., 1918, 28, p. 759.

²⁶ Ann. de l'Inst. Pasteur, 1919, 33, p. 557.

increased by passage from animal to animal, probably in a manner somewhat similar to the one described by Ten Broeck.²⁷

Even guinea-pigs were endangered, though spontaneous cases were not observed by us. Ingestion of 250,000 organisms was fatal for guinea-pigs varying in size from 400-500 gm. in from 4-8 days, using the recently isolated culture. The necropsy findings differed in no respect from those commonly noted in rodent paratyphoid or pseudotuberculosis. The virulence of cultures 6 to 12 months old diminished similarly for guinea-pigs as for rabbits, and larger doses were necessary to produce an infection. Daily feeding of a whole growth of an agar slant (culture 18 months old) on minced carrots for 3 weeks produced a chronic disease with positive cultural findings in the spleen, mesenteric lymph nodes and liver. It was noted that white mice that accidentally had access to the remains of the carrot mixture succumbed to an acute infection (10-12 days), with typical lesions and positive cultural findings in all organs. At least for this species of animal the organism had retained a considerable degree of virulence.

The feeding experiments on rabbits furnished a series of observations that appear to be of considerable importance and therefore deserve some consideration. Contrary to our findings in the intravenously inoculated rabbits in which a diphtheric or necrotized cholecystitis with positive paratyphoid bacilli findings were recorded, the rabbits fed and successfully infected with the same organism of the same age did not show a cholecystitis. Invariably the cultures obtained from the bile were sterile. These results are fully in accord with those described for the spontaneous cases. We also recall in this connection that the recent statistics of Hübener²⁸ and Herxheimer¹¹ on acute paratyphoid fever infections in man fail to mention pathologic changes in the gallbladder. One is probably justified in assuming that in their cases, at least, this organ was not diseased. These facts may contribute some information concerning the pathogenesis of cholecystitis due to organisms of the typhoid-paratyphoid group in rabbits free from coccidiosis or other infections of the liver. One fact stands out preeminently, namely, irrespective of the number of paratyphoid bacilli in the blood and tissues at the time of death, the animals that received their infection by way of the intestinal tract did not show a cholecystitis nor even bacilli in the bile so perfectly suited for their proliferation. The gallbladder wall may on enrichment in broth be found to be infected, which is not surprising when the blood stream is teeming with specific organisms.

It will be the purpose of another series of papers to discuss in detail the manner in which typhoid bacilli, for example, reach the gallbladder and bile in rabbits, but we can already state that an ascending invasion via the common and cystic duct has not been observed in our studies. On the other hand, there is conclusive evidence at our disposal which indicates that the infection is descending. Typhoid or paratyphoid bacilli inoculated intravenously in certain doses, which vary according to the virulence of the organisms, the size of the animal and other factors to be considered elsewhere, appear in the common duct bile in the first 10-15 minutes after the injection. The bacteria reach the bile by way of the biliary passages and proliferation in this secretion or the constant feeding of the same from liver foci will lead to a more or less prolonged sojourn of the organisms with secondary inflammatory involvement of the gallbladder wall. In our experience another route of bile infection is occa-

²⁷ Jour. Exper. Med., 1917, 26, p. 437.

²⁸ Fleischvergiftungen und Paratyphusinfektionen, 1910, p. 118.

sionally noted in rabbits, namely, the embolic invasion of the capillaries of the gallbladder wall. This mechanism of invasion is particularly common when virulent strains in large doses are used. The histologic picture of the gallbladder wall of the intravenously inoculated rabbits in our pathogenicity series suggested such a hematogenous infection as the most likely mode leading to the diphtheric or necrotized cholecystitis. Bacterial thrombi are found in the capillaries at the base of the fundus of the gallbladder mucous membrane. These foci are surrounded by areas of necrosis or leukocytic infiltration. Such portions of the mucosa may be covered by an intact epithelium. In the majority of cases, however, the complete destruction of the entire mucous membrane, muscularis and serosa does not permit an insight into the cycle of events leading to the complete destruction of the gallbladder. The difference in the gallbladder lesions between the spontaneously infected, the experimentally fed and the intravenously inoculated rabbits may therefore be readily explained as follows: Paratyphoid bacilli intravenously introduced rapidly accumulate in large numbers in the liver capillaries; the gallbladder as an appendix of this organ and connected by accessory portal veins (Violle²⁰), receives a proportional share in its small blood vessels. In the parenchyma of the liver the characteristic necroses and cellular proliferation develop as a result of the multiplication of the retained bacteria; in the gallbladder wall a similar process leads to a diphtheric necrosis of the mucous membrane with subsequent invasion of the bile. The latter may also receive bacteria simultaneously from the biliary passages. Intensive proliferation in this secretion leads to additional destruction of the mucous membrane from within and in turn may cause complete necrosis, even beginning perforation of the gallbladder wall. In the spontaneously infected or in the fed animals the number of organisms that reach the liver is comparatively small and the invasion gradual. The protective properties of the liver (also seen in immunized rabbits inoculated intravenously) prevents the indirect passage of paratyphoid bacilli from the hepatic veins to the biliary capillaries. A few bacteria reaching the bile are probably rapidly discharged in the hepatic duct bile, which is continuously flowing as result of the toxic enteritis, and in all probability no organisms reach the cystic bile where multiplication is most suitable. This reconstruction of the events is borne out by the cultural studies in this and other series of animals studied for the same purpose. The gallbladder bile, the duodenal and jejunal contents were free from paratyphoid bacilli. Such bacilli were found only around or below the diphtheric or ulcerated agminated follicles and in the region of the sacculus rotundatus and appendix. On the other hand, the gallbladder wall of the fed or spontaneously infected animals was found to contain a few paratyphoid bacilli; this is not surprising when we realize that on one occasion 720 organisms were present in 1 c.c. of heart blood. A direct, heavy invasion of the gallbladder wall and its blood vessels was made impossible on account of the gradual infection of the liver from the portal system. Consequently, the formation of bacterial emboli primarily responsible for the diphtheric inflammation was eliminated. These observations furthermore show that in the rabbit at least a portal septicemia with rabbit pathogenic virulent and invasive paratyphoid bacilli does not necessarily lead to a cholecystitis or to an infection of the bile. On the other hand, the intravenous injection of the same organism in comparatively small numbers causes a hematogenous invasion of the gallbladder and bile by way of the biliary passages on one hand and by way of the capillary thrombi in

²⁰ Ann. de l'Institut. Pasteur, 1912, 26, p. 384.

the wall on the other. In case the animal has lesions of coccidiosis or the organism possesses specific selective organotropic properties for the gallbladder, as was shown by Fraenkel and Much,³⁰ localization in this organ may occur following any method of infection. These facts have in our opinion a bearing on the typhoid-paratyphoid carrier problem in laboratory animals, which we will discuss elsewhere more in detail.

TOXIN PRODUCTION

Smith and Ten Broeck³⁰ observed that bacteria of the typhoid-paratyphoid group are capable of producing highly potent toxins for rabbits in peptonized sugar-free veal broth plus 0.1% glucose incubated in shallow layers. One of us (K. F. M.) several years ago conducted a large number of experiments on dogs and rabbits with the toxic filtrates from *B. abortus equinus* prepared according to the procedure given by these writers. The results were rather irregular and at that time it was impossible to investigate the factors responsible for the inconstant results. A series of tests conducted with the paratyphoid bacilli isolated from the rabbit apparently offer an explanation for the failures recorded. It was found that aside from the depth of the layer of fluid in which the cultures were grown, namely, not more than 2 cm., the reaction of the medium is an important factor. We originally prepared the substratums for the production of toxic filtrates strictly according to the formula of Smith and Ten Broeck with Witte's peptone, adjusting the fluid to an initial reaction of 1% acid to phenolphthalein. The final product had a H-ion concentration of P_H 7.2-7.4. Several batches of mediums with this reaction were tried. Invariably the toxic filtrates of 7-day old cultures, inoculated in doses of 1 cc per kilogram of weight, produced temporary restlessness, labored breathing followed by slight stupor, passage of urine and feces and loss of weight of from 75-100 gm., but never death. Experiments with other toxins and particularly the generally known fact that diphtheria toxin is only produced at a P_H of from 7.8-8.4 suggested some experiments with mediums adjusted to such a reaction. Powerful toxic filtrates, even from 48-hour cultures, were obtained in a broth with a P_H of 8.4. For illustration of this statement, we cite in detail one experiment:

Rabbit 1749, weighing 2,350 gm., received at 2:30 p. m., 2.5 cc of filtrate of a 72-hour old broth culture of paratyphoid bacilli 1371 (initial reaction of medium P_H 8.4) into an ear vein. At 4:30 was drowsy and breathing labored with a snuffling noise, hind leg extended, eyes dull and partially closed. At 9 p. m. the rabbit was found dead. The necropsy showed hemorrhages in the abdominal and pelvic lymph nodes, engorgement of the liver, and spleen, petechiae on the serosa and mucous membrane of the stomach and the duodenum. There were 2 cc of fluid in each pleural sac; the lung was slightly congested and moist; there were intense congestion in the trachea and petechiae on the pericardium; the blood was thick and tarry.

The course of the intoxication and the necropsy findings were practically identical in all the animals injected with these toxins and in a general way differed in no respect from the description given by Smith and Ten Broeck and Mulsow³¹ for various representatives of the typhoid-paratyphoid group.

In connection with some other problems, we were interested in the possibility of producing toxic organ filtrates according to the procedure of J. T.

³⁰ Jour. Med. Research, 1914, 31, p. 523.

³¹ Jour. Infect. Dis., 1919, 25, p. 135.

Parker.³² This writer demonstrated that the liver of rabbits inoculated with cultures of *B. typhosus* or *B. prodigiosus* under certain conditions contain a toxic substance extractable with salt solution. When the toxic extracts are injected intravenously into normal rabbits these animals develop symptoms resembling those of anaphylactic shock and succumb. We have confirmed these observations with the *B. typhosus*, but thus far we have been unable to obtain toxic liver extracts of rabbits injected with one half to one slant of our paratyphoid bacillus. Even by producing the most favorable conditions by rendering the animal very sick in 6-10 hours after the injection of one half slant of a young culture no liver poison was elaborated that would kill small rabbits in the chosen dose of 10 c.c. of extract. Our paratyphoid bacillus does, however, behave in a similar manner to a number of other paratyphoid strains tested by the same technic. Certain immunity tests to be reported elsewhere suggest that the toxemic manifestations of paratyphoid infections in rabbits are different from those constantly observed in these animals injected with typhoid bacilli. A progressive septicemia with a high bacterial count of the blood and tissues precedes the death of paratyphoid rabbits. On the other hand, the fatal issue in rabbits injected with typhoid bacilli is frequently accompanied by a comparatively low bacterial blood and tissue count. It is not unlikely that the greater bactericidal power of the blood and tissues of the rabbit for typhoid bacilli is responsible for the rapid destruction of viable bacteria and the production of a large amount of poison which causes the death of the animals. These bacteriolytic forces are apparently not operative to the same degree in paratyphoid infection in rabbits, and poisons are not elaborated in the tissues; or the liver is able to neutralize large amounts of toxin as they are formed, and a quantity which exceeds the detoxicating threshold is never produced. In both instances, the poison naturally cannot be demonstrated in the salt extracts. Whether these interpretations are the correct ones or whether they only represent one phase of a more complex mechanism is at present the subject of a more extensive investigation.

GENERAL SUMMARY

The foregoing study demonstrates the occurrence of spontaneous paratyphoid *B.* infections in rabbits. Unfortunately it was impossible to determine the origin of the disease; the dealer supplying the infected rabbit, which initiated the epidemic described, had disposed of his stock when the nature of the malady was properly established. It would have been of considerable practical importance to have known whether the existence of carriers was responsible for the development of the acute cases. The possibility that certain types of abortion in rabbits may be caused by paratyphoid bacilli and that animals in such a condition may disseminate the infection cannot be dismissed in the light of our observations. It must also be the question to be decided whether or not the causative strain was of guinea-pig or of mouse origin. The fact that the dealer did not raise guinea-pigs or mice and the extreme rarity of paratyphoid *B.* infections in rabbits in

³² Jour. Exper. Med., 1918, 28, p. 571.

contrast to the rather ubiquitous distribution of this malady in guinea-pigs and mice, does not lend much support to this conception. Rabbits are frequently exposed to pseudotuberculosis of guinea-pigs. Repeatedly diseased animals have been introduced into our cages, and before the infection was recognized, numerous rabbits have been exposed to their discharges without causing a paratyphoid epidemic. Moreover, the agglutination tests conducted with the strains isolated from a series of different guinea-pig epidemics in our laboratory failed to indicate a close relationship to the rabbit-paratyphoid organism. The determinations of the source of paratyphoid infections must therefore be reserved for future investigations.

Our observation, which shows how readily an epidemic among laboratory rabbits may get started, should be a lesson to all laboratory workers. It is obvious that careful necropsy and complete cultural examination should be performed on all stock animals, even if the circumstances of their death suggest only an accidental infection. It has been our policy to keep complete records of the origin of the animals received and to place the same in experiments only after a strict quarantine in isolated cages for at least two weeks.

This discussion cannot be closed without a brief consideration of the relationship of our paratyphoid organism to so-called food poisoning. Although MacConkey⁸ reports cases of food poisoning in which rabbit meat was involved, it is definitely shown by the serologic tests that our bacillus does not antigenically belong in the human paratyphoid or *B. schottmülleri* group. Yet the ability of this organism to evolve a potent toxin should be considered in any attempt to explain outbreaks of food poisoning in which rabbit flesh is a part of the suspected meal. Even if the reports on meat poisoning caused by rabbit meat are limited to the one by MacConkey, it would be most unwise to say that this food cannot cause disease in man and to use this as an argument for the safety of man against the animal paratyphoid bacilli. Individual susceptibility may play an important rôle and a nonpathogenic organism may be readily transformed into a potent factor of gastro-enteritis.

PARATYPHOID BACILLI FROM CHICKS

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During several years past we have experienced many serious losses among chicks, wherein the lesions have been fairly constant, and the preliminary bacteriologic examinations have shown the presence of paratyphoid bacteria. Since there was considerable doubt as to the exact nature of the organisms concerned, it was deemed advisable to undertake an intensive study of organisms from a number of outbreaks. Considerable work has been done by others in connection with the etiology of bacillary white diarrhea in chicks, but for the most part such work has been with old laboratory strains of *Bact. pullorum* and related organisms.

Our study comprises 21 strains of paratyphoids freshly isolated from 21 outbreaks of highly destructive disease among chicks. For comparison there are included 2 strains from ovaries of mature hens; one official strain each of *Bact. gallinarum*, *Bact. pullorum*, *B. enteritidis*, *B. paratyphosus* A, *B. paratyphosus* B, and *B. typhosus*. The chicks attacked in these outbreaks were usually from 2 to 4 days old. The lesions most constantly found in the chicks were slight enlargement of the liver together with petechiae and necrotic foci. These liver lesions were found in at least 90% of the chicks from which paratyphoid bacteria were isolated, and were rarely found in chicks from which paratyphoids could not be isolated. Pneumonia was found in about 70% of the affected chicks. Quite frequently yellowish, friable nodules were found in the lungs. Occasionally these nodules were found in organs other than the lungs. Abscesses in the cecums were found in about 8% of the cases.

Bacteriologic Study.—No new technic has been introduced in this study, but certain methods of recent origin were used, and it is believed that interesting information, relative particularly to *Bact. pullorum* and *Bact. gallinarum*, is first reported herein on recently isolated strains. Control tests were made in all mediums on authentic strains of *Bact. gallinarum* G 1, *Bact. pullorum* P 1, *B. paratyphosus* A-A 97, *B. paratyphosus* B-B 96, *B. enteritidis* E 117, and *B. typhosus* T 114.

Morphology.—All of the 29 strains were gram-negative, and were of a size and form common to the paratyphoid bacilli. All of our strains, except strain P 14, were identical with the type strain of *Bact. pullorum* P 1, and commonly exhibited a tendency toward bipolar and peripheral staining.

Motility.—Control strains A 97, B 96, E 117, and T 114, and our strain P 14, were actively motile; control strains G 1, P 1, and our other 22 strains were nonmotile. The organisms of strain P 14 appeared to bear a single polar flagellum, although some cells seemed to have a second one attached at the same pole. Flagella could not be demonstrated on any of our other strains. Typical peritrichial flagella were readily demonstrable on the control strains A 97, B 96, T 114, and E 117, but were not demonstrable on the control strains G 1 and P 1.

Cultural Characteristics.—Twenty-two of our strains may be grouped for discussion, but strain P 14 apparently represents a new type, and will be treated separately.

No indol reaction could be demonstrated in any of our 23 strains, nor in any of the control strains.

All of our 23 strains produced H_2S in lead acetate agar. A positive reaction was also obtained for all control strains except *B. paratyphosus* A-A 97.

All strains, including control strains, gave an initial acidity in litmus milk which lasted over a varying period, returning to neutral then alkaline in the following order: *B. enteritidis* E 117 alkaline at 3 days; *B. paratyphosus* B-B 96 at 8 days; *Bact. gallinarum* G 1 at 13 days; *B. typhosus* T 114 at 25 days; *B. paratyphosus* A-A 97 at 35 days; *Bact. pullorum* P 1 at 35 days.

Of our strains, P 14 became alkaline after 3 days. The remaining 22 strains retained their acidity for at least 70 days. At 72 days one strain was perceptibly alkaline; 9 strains became alkaline between 72 and 95 days; and the remaining strains were neutral or acid at 96 days.

Milk cultures of control strains became translucent and opalescent in the following order: *B. enteritidis* E 117, *B. paratyphosus* B-B 96, and *B. typhosus* T 114 at 53 days; *Bact. gallinarum* G 1 at 90 days; *B. paratyphosus* A-A 97 at 104 days; *Bact. pullorum* P 1 remained opaque at 107 days. Of our strains, P 14 became translucent and opalescent at 72 days, and the remaining 22 strains were still opaque at 107 days.

Considerable work has been done on the subject of fermentation of carbohydrates in the effort to differentiate *Bact. pullorum* from *Bact. gallinarum*. The method employed in these studies was, for the most part¹ based on titration at varying periods of extract or infusion broth cultures containing 1% of the desired carbohydrate. Krumwiede and Kohn² substituted the use of the Andrade indicator in the sugar broth for titration.

We have found that the use of 1% sugar-serum-water plus the Andrade indicator, as advocated by Krumwiede, Kohn, and Valentine,³ possesses certain advantages over titration in that immediate and frequent observations may be made; gas production is readily detected; the slightest initial acidity may be noted; and reducing ability may be distinguished by the decolorization of the coagulated serum. Another indicator, "C-R,"⁴ was tried, but it possessed no advantage over the Andrade indicator.

¹ Hadley, P., Rhode Island Bull., 178, 1918; Smith, T., and Tenbroeck, C., Jour. Med. Res., 1914, 26, p. 547; Goldberg, S. A.: Jour. Amer. Vet. Med. Assoc., 1917, 51, p. 203.

² Jour. Med. Res., 1917, 31, p. 511.

³ Jour. Med. Res., 1918, 38, p. 89.

⁴ Bronfenbrenner, Davis, and Morishima, Jour. Med. Res., 1919, 39, p. 346.

As a preliminary test for native sugar in the serum, tubes of serum-water (1 part serum, 4 parts distilled water, plus 1% of the Andrade indicator) were prepared without the addition of any sugar. These were inoculated with the 6 control strains and incubated for 3 days. No trace of acidity was perceptible at any time, and as all tests were made in the same lot of serum-water, fermentation subsequent to the addition of a known sugar could therefore be reasonably ascribed only to the presence of such sugar.

Control strains G 1, P 1, and T 114, and 12 of our 23 strains gave acid, coagulation, and no gas in glucose; control strains B 96, A 97, E 117, and 11 of our strains gave acid, coagulation, and varying amounts of gas; control strains B 96 and E 117, and our strain P 14 completely decolorized the indicator. The test was immediately repeated on all strains which produced no gas, and identical results were obtained.

All strains were then tested in fermentation tubes of 1% glucose infusion broth (rendered sugar-free by *B. coli*) plus Andrade indicator. Control strains G 1, P 1, T 114, and 5 of our strains gave acid with no gas. All other strains gave acid and gas in amounts varying from a bubble to 20%. In comparing the results of this test in broth with the above tests in serum-water we find that 2 of our strains did not produce gas in glucose broth but did in serum-water; 8 of our strains produced gas in glucose broth but did not in serum-water; control strains G 1, P 1, T 114, and 3 of our strains were constantly negative for gas.

All strains, including controls, were negative for acid and gas in lactose infusion broth. In lactose serum-water all strains, including controls, gave a slight but distinct acid reaction at 4 to 8 hours. At 18 hours B 96, A 97, and E 117 were colorless, while the reaction persisted in all of the others without intensifying for 24 to 48 hours. In no case was the acidity sufficient to coagulate the serum. This test was repeated with identical results. Whether this slight reaction represented an actual fermentation of lactose, or of a product of hydrolysis due to sterilization is not known.

The results with dextrin were unsatisfactory; control strains G 1, A 97, B 96, T 114, and E 117, all produced undoubted acidity, but not of sufficient strength to coagulate the serum. In the absence of coagulation the determination of gas was also unsatisfactory. However, *Bact. pullorum* P 1, commonly described as unable to ferment dextrin, and all of our strains produced slight but distinct acidity. The factor of hydrolysis, however, may again have entered into these results.

All strains were negative on two tests in saccharose and salicin. Control strain B 96 and our strain P 14 produced acid, gas, and coagulation in inosite, and decolorized the indicator, thus conforming to the classification of a true *B. paratyphosus*. *B. B. enteritidis* E 117 and all other strains gave an entirely negative reaction. With dulcitol control strains B 96, E 117, and our strain P 14 gave acid, gas, and coagulation, and decolorized the indicator. Control strain A 97 gave acid, gas, and coagulation without decolorization; control strain G 1 gave acid, coagulation, and some decolorization, but no gas; control strain P 1, T 114, and all of our strains, except P 14, showed no fermentation.

Control strains B 96, E 117, and our strain P 14 gave acid, gas, coagulation, and complete decolorization in xylose; control strain T 114 gave acid and coagulation, but no gas nor decolorization; control strains A 97, G 1, P 1, and all of our strains, other than P 14, were uniformly negative.

Control strains B 96, E 117, and our strain P 14, gave acid, gas, coagulation, and decolorization in mannite; control strain A 97 gave acid, gas, and coagulation, but no decolorization; control strains G 1, P 1, and T 114 gave acid

and coagulation, but no gas nor decolorization. Of our strains, 4 gave acid and coagulation, but no gas nor decolorization; all others gave acid, gas, and coagulation, but no decolorization.

In comparing the fermentation of mannite with that of glucose we find that, of the 12 strains which did not produce gas in glucose, 8 produced gas in mannite; 4 strains were negative for gas in both sugars.

Control strains B 96, E 117, and our strain P 14 produced acid, gas, coagulation in levulose, and decolorized the indicator; control strain A 97, and 10 of our strains, other than P 14, produced acid, gas, and coagulation, but no decolorization.

In galactose the control strains, and our strain P 14, behaved exactly as in levulose; on the first test one of our strains, other than P 14, produced acid, gas, and coagulation, with no decolorization; 21 strains produced acid and coagulation, but no gas nor decolorization. This test was immediately repeated, and gas was obtained from 3 strains which were negative on first test. Two weeks later the test was again repeated and 3 strains which produced gas on the second test showed gas, while the strain which formed gas on the first test failed to show gas on the third test.

In arabinose control strains B 96, A 97, E 117, G 1, P 1, and our strain P 14 behaved as in levulose and galactose; control strain T 114 produced only slight acid, with no gas nor coagulation; 3 of our strains produced acid, gas, and coagulation, and the other 19 produced acid and coagulation but no gas.

In view of the statements of other investigators^{1, 2} regarding the differentiation of *Bact. gallinarum* from *Bact. pullorum* on the grounds of ability or inability to ferment maltose, we were surprised to find a perceptible acidity at 4 hours in all tubes of maltose serum-water inoculated with our 23 strains. At 18 hours the medium was coagulated, and 16 strains had produced gas. The test was immediately repeated with identical results.

The test was again repeated, this time in fermentation tubes of infusion broth, previously rendered sugar-free by a strain of *B. coli*. One per cent. maltose and 1% Andrade indicator were added to the medium. Our results conformed to those of other investigators; control strains G 1 and T 114 developed strong acid but no gas; control strains A 97, B 96, E 117, and our strain P 14 developed acid and gas; control strain P 1, and all of our strains, other than P 14, gave no evidence of acid nor gas on 5 days' incubation.

After consideration of this conflicting evidence the test was again repeated in serum-water, using the "C-R" indicator. Our first findings were confirmed; this time all of our strains but one formed gas. For several strains this was the first evidence of gas production in any medium.

The factor of hydrolysis must again be considered, and we do not attempt to explain why maltose infusion broth and maltose serum-water, autoclaved alike at 15 pounds for 15 minutes, should give such contradictory results. The serum-water for the second test was sterilized in an Arnold sterilizer in an attempt to avoid hydrolysis. Whether or not the intermittent method offers any advantage over autoclaving, the results on inoculation were the same. Krumwiede and Kohn² have noted fermentation in maltose broth inoculated with strains of *Bact. pullorum* and incubated over a period of 16 to 40 days. They, too, consider the extent to which hydrolysis may have influenced their observations.

It has been observed by Hadley¹ that some organisms produce gas in sugar infusion broth but do not produce gas in sugar extract broth, and that acid production is similarly affected. It is possible that serum-water, notably an ideal medium, may in the same way influence fermentation by organisms that possess a low avidity for the given sugar.

Agglutination Tests.—The agglutinative affinities of this group of organisms have been so thoroughly studied by Krumwiede and Kohn, Smith and Tenbroeck, Hadley, and others, that we have used the test not in an attempt to differentiate, but to group the organisms included in our study. We have found that, of our 23 strains, 20 strains from chicks and the two strains from diseased ovaries of hens are apparently identical with the type strain of *Bact. pullorum* P 1. While cross agglutination with *Bact. gallinarum* serum and antigen was observed to a high titer, there was a well-marked distinction between our strains and the control strain of *Bact. gallinarum* G 1.

Strain P 14, which gave cultural reactions identical with those of a true *B. paratyphosus* B, was peculiarly distinct agglutinatively from the control strain B 96, and is apparently a newly observed type.

SUMMARY

In this study of freshly isolated paratyphoids from chickens we have found 20 strains from chicks showing lesions of bacillary white diarrhea, and 2 strains from diseased ovaries of hens, to be identical with a type strain of *Bact. pullorum*. One new type was encountered which closely resembled the *B. paratyphosus* B type, but which was agglutinatively distinct, and appeared to have commonly only a single polar flagellum; some cells doubtfully displayed a second.

Constant lesions were associated with the presence of these organisms; enlargement of the liver, together with petechiae and necrotic foci in the liver, and pneumonia and yellow friable nodules in the lungs were characteristic. Abscesses in the cecum were found occasionally.

One strain isolated from a chick fermented the proper sugars but did not produce gas from any. All other strains produced gas from one or more sugars.

No correlation could be shown in gas production from various sugars; some strains produced gas from glucose and not from mannite; the behavior of others was exactly the reverse. Repeated tests were necessary to demonstrate gas production by some strains.

All of our strains and the control strain of *Bact. pullorum*, usually regarded as unable to ferment maltose, produced acid and gas in 1% maltose serum-water. Maltose infusion broth was not fermented.

OCULAR REACTIONS IN ANAPHYLAXIS

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The anaphylactic state appears to concern practically every part of the body, and I have made observations especially of the anaphylactic reactions of the eye and of the structures closely associated with it. Most of the studies so far made in this respect have not been thorough enough, and in order to accomplish the purpose of my work, I limited myself to the study of the reactions of guinea-pigs to horse serum.

Schultz¹ studied the reactions of the smooth muscle of the intestine and of the blood vessels, etc., in anaphylaxis, and Dale² the reactions of the uteri muscle and the lung of guinea-pigs. These investigators observed an increased reactivity of smooth muscles in sensitized animals, which was confirmed by Friedberger and Kumagai.³ It was therefore natural to study the reactions of the smooth muscles of the eyelids and the iris, the blood vessels of the lids, the conjunctiva, the fundus and reactions of the lacrimal and Harder's glands, to the application of horse serum locally, as well as after general administration. At the same time, certain general phenomena were closely observed. I made a special effort to study the local ocular phenomena.

The animal was kept quiet in a dark room and all confusing stimulation was avoided carefully. Both eyes were illuminated symmetrically with an electric light, 40 w, 115 v, from a distance of 30 cm. in front of them. The width of the lid and of the pupil were measured by a palpebro-pupillometer that I had made, figuring 15 light and 15 dark circles of from 1.0 to 8.0 mm. in diameter, and 0.5 mm. apart, arranged in regular parallel rows, both vertical and transverse lines crossing in the center of the square of oblong quadratic side lines, the side and crossing lines being marked in millimeters. The light circles were used for the measurement of the width of the lids generally, also of the width of the pupils of the albinotic eyes; the dark circles were used for the measurement of dark pupils; the side and crossing scales served as controls. The pupils were measured after adaptation of the retina to light for 5 minutes.

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¹ Jour. Pharmacol. and Exper. Therap., 1910, 1, p. 549; Bull. Hyg. Lab., 1912, No. 80.

² Jour. Pharmacol. and Exper. Therap., 1913, 4, p. 167.

³ Ztschr. f. Immunitätsf. u. Exper. Therap., 1914, 22, p. 269.

The iris was examined by a magnifying lens under electric light and when necessary by oblique illumination.

The examination of the fundus was made by indirect and direct methods, using the De Zeng's electric ophthalmoscope.

Effect of Instillation of Normal Salt Solution into the Conjunctiva of Normal Guinea-Pig.—The animal was a white male with pink eyes; one drop of 0.9% salt solution was dropped into the left eye 3 times at intervals of 30 minutes. The results (table 1) indicate that a drop of salt solution dropped into the conjunctival sac of a normal albino guinea-pig may cause a slight contraction of the pupil (0.5 mm.). A second drop was followed by a stronger closure of the lid but without any change in the pupil, and a third drop had the same effect. In this case the closure of the lid and the contraction of the pupil were of course reflex.

TABLE 1
LID AND PUPIL REACTION TO INSTILLATION OF SALT SOLUTION

Time in Minutes		Left Eye		Right Eye (Control)	
		Lid Fissure in Mm.	Pupil in Mm.	Lid Fissure in Mm.	Pupil in Mm.
Before		6	3	6	3
After First Instillation	1	5	2.5	6	3
	4	5.5	3	5.5	3
	10	5	2.5	5.5	3
	12-17	5.5	3	5.5	3
	20	5	2.5	5.5	2.5
	25-20	6	3	6	3
After Second Instillation	1	2	2.5	5.5	3
	3	3	2.5	5.5	3
	5-7	3	2.5	4	3
	10-12	5	3	5	3
	15-19	5.5	3	5.5	3
	20	3.5	2.5	4	3
	22	4	3	4	3
	25	5	3	5.5	3.5
	27	5	3	5	3
	30	5.5	3	5.5	3
After Third Instillation	1	3	3	5.5	3.5
	3	2	3	5	3
	5	3	3	4	3
	7	5	3	5	3
	10	4	3	4	3
	12	4.5	3	5	3
	15	5	3	5	3

The Ocular Reactions of a Normal Guinea-Pig to Normal Unheated Horse Serum Dropped on the Conjunctiva.—The animal was a male, brown and white, the iris dark brown, the fundus strongly pigmented. It was kept in a dark room and the eye illuminated from the left side in this instance. When the eye had become adapted to the light, one drop of unheated normal horse serum was placed on the conjunctiva of the same eye 3 times at intervals of 30 minutes. The other eye was kept covered during this time. The results of the measurements are shown in table 2.

I have observed that the eyes of guinea-pigs do not react together, and it may not be possible to avoid certain irregularities in the results.

In this experiment the first drop of normal horse serum caused opening of the lid and dilatation of the pupil in one minute. The opening of the lid was at its maximum at first and lasted for 7 minutes. The dilatation of the pupil reached its maximum in 7 minutes and continued for about 10 minutes. The second drop of horse serum caused narrowing of the lid and increased dilatation of the pupil, the dilatation reaching its maximum in 3 minutes and continuing for 17 minutes. The third drop seemed to be without any effect. Until 2 hours later the lids and pupil of the eye experimented on remained more contracted than in the control eye.

TABLE 2
LID AND PUPIL REACTION OF NORMAL GUINEA-PIG TO NORMAL HORSE SERUM

Time in Minutes		Left Eye	
		Lid Fissure in Mm.	Pupil in Mm.
Before		5:5	3.5
After First Instillation	1-3	6	3.75
	5	6	4
	7	6	4.5
	10	5.5	4
	11	5.5	3.5
	13-17	5	3.5
	24-27	4.5	3.5
	30	5	3.5
After Second Instillation	1	4	4
	3-5	3.5	4.5
	7	4	4
	9	4	3.75
	12	4	3.75
	17	4	3.75
	25	5	3.25
	30	4	3.25
After Third Instillation	1	4	3.25
	5	3.5	4
	10-25	4	3
	30	2	3

Ocular Reactions in a Normal Albino Guinea-Pig to Unheated Normal Horse Serum.—The animal was male, wholly white; the fundus was not pigmented. One drop of normal horse serum was introduced into the left eye 3 times, the intervals being 15 and 45 minutes. In this case the response was more prompt than in the previous animal, the dilatation of the pupil reaching its maximum in 2 minutes. The second and third drops were followed by a more steady widening of the lid space and dilatation of the pupil, and this was succeeded by contraction lasting for about half an hour. This experiment shows that in the albino guinea-pig, the lid, as well as the pupil, reacts vigorously to repeated instillation of horse serum. Probably the differences between the albino and pigmented animals were partly individual and partly due to the differences in the content of pigment.

Ocular Reactions of Sensitized Guinea-Pig to Unheated Normal Horse Serum Dropped Into the Conjunctival Sac.—A male guinea-pig, practically wholly white, was injected Nov. 20 with 0.01 c.c of normal horse serum subcutaneously. About 17 weeks later normal unheated horse serum, after having been warmed in the incubator, was dropped into the left eye 3 times at intervals of 30 minutes. In this test both eyes were illuminated with the same light and

in the same way. All eye phenomena and general symptoms were observed carefully. The results are given in table 4; the first drop caused an immediate opening of the lid and a vigorous dilatation of the pupil, the maximum being reached at once; 13 minutes later the lid and the pupil contracted. The second drop caused a slower but longer mydriasis, but had no effect on the width of the lid. The third drop was followed by narrowing of the lid and of the pupil. There was a noticeable congestion of the palpebral and ocular conjunctiva, of the margins of the cornea and of the iris, the lids being somewhat swollen, and the conjunctiva remarkably edematous; at the same time there was marked lacrimation. The congestion and edema began 36 minutes after the first drop and increased after each additional drop. It should be noted that a moderate congestion of the papillae also developed in the treated eye. The animal was restless and rubbed its nose; there were abundant fecal and urinary discharges. It is therefore evident that in this case the instillation of horse serum into the conjunctiva of a sensitized guinea-pig gave rise to both local and general anaphylactic reactions. About 4 hours after the first drop of horse serum was put in the eye, 6 c c of horse serum was injected into the peritoneal cavity and the eye and other reactions observed in the same way as before for about 37 minutes. For about 8 minutes the animal was quiet, his head reclining to the left, both eyes being watery, especially the left; in 13 minutes, he scratched his nose continually; in 14 minutes, there was a whitish discharge from both eyes, more marked in the left; in 15 minutes, the rectal temperature was 39.8 C. and respiratory frequency increased; in 17 minutes, there was circumcorneal injection, especially marked in left eye, and the body muscles were feeble; after 20 minutes the animal fell suddenly; he had convulsions of the forelegs and both eyes opened to 6 mm.; both pupils dilated to 4.5 mm.; in 23 minutes, both forelegs went through violent scatching movements; after 25 minutes the animal became quiet, lying on the belly; micturition; in 27 minutes lid fissures were 5.5 mm., pupils 3.5 mm.; in 30 minutes, the fundi were a little congested, but no hemorrhage; in 35 minutes, rectal temperature was 39.4 C.; he was a little dyspneic and at times had clonic convulsions; in 37 minutes the animal arose; the width of both lids was 5.0 mm., of both pupils 2.5 mm.; circumcorneal injections, chemosis and lacrimation appeared.

The animal was chloroformed; both lids closed, respiration ceased; he had clonic spasms; the lids were 3.0 mm. in width, the pupils 3.5 mm. in 6 minutes; death occurred in 8 minutes; the width of the lids was 4 mm., of the pupils, 5.5 mm.

After death the brain and spinal cord were markedly congested, also the mucous membranes of the eye and nose; the lungs were somewhat distended, with a few hemorrhage spots; liver, spleen and intestines were congested; other organs were normal.

Reactions to Heated Normal Horse Serum Dropped Into the Conjunctival Sac of a Sensitized Guinea-Pig.—The animal was a wholly white male; there was no pigment in the fundus. It was sensitized by subcutaneous injection of 0.01 c c of normal horse serum heated at 56 C. for 30 minutes and tested 110 days later by dropping one drop into the left conjunctival sac. The serum was warmed in the incubator before being placed in the eye, which was done 3 times, one drop at a time, with an interval of one hour between the first and second time and 30 minutes between the second and third time. After observing the changes in the eyes, 6 c c of heated serum were injected intraperitoneally, and the animal was killed with chloroform (table 5).

There was a slight contraction of the lids and of the pupil immediately after the instillation of the heated serum, probably due to reflex action; from 11 to

TABLE 3
LID AND PUPIL REACTION TO NORMAL HORSE SERUM IN A NORMAL ALBINO GUINEA-PIG

Time in Minutes		Left Eye		Right Eye (Control)	
		Lid Fissure in Mm.	Pupil in Mm.	Lid Fissure in Mm.	Pupil in Mm.
Before		5	3	5	3
After First Instillation	2	6	3
	5	6.5	3
	7	7	3
	10	5	3	5	3
After Second Instillation	15	5.75	3.75	5.5	3.5
	17	5	4.5	4	3.5
	22	4.25	4	4	3.5
	27	5	3	4.5	2.5
	35	5.5	4	5	3.5
	40	6	4	5.5	3.5
After Third Instillation	3	6	4.5	5.5	3.5
	5	5	4.5	5	3.5
	10	4.5	4.5	5	4
	17	5.5	4.5	5.5	4
	25	5.5	4.5	5.5	4.5
	30	6	5	5	4
	45	4.75	4	4.5	3.5
	66	5	4	5	3.75

TABLE 4
LID AND PUPIL REACTION OF A SENSITIZED GUINEA-PIG TO NORMAL HORSE SERUM INSTILLED IN EYE

Time in Minutes		Left Eye (Instilled)		Right Eye (Control)		Remarks
		Lid Fissure in Mm.	Pupil in Mm.	Lid Fissure in Mm.	Pupli in Mm.	
Before		4	2.5	4	2.5	
After First Instillation	1.5	5.5	4.5	5	3	Exelited, seratching Left eyelids and conjunctive congested and swollen, circumcorneal injection and hypersecretion
	3	5	3	5	2.5	
	5	5	2.5	5	2.5	
	7	5	2.5	5	2.5	
	10	5	3	5	4	
	13	3.5	2	4	2.5	
	15	3	2	3	2	
	20	5	4	5	4	
	25	4.5	3	4.5	3	
	30	3	2.5	3	2.5	
After Second Instillation	37	5	3	5	3	Left external congestion and chemosis increased; iris and optic disk congested
	3	4	3.5	4	3	
	5-7	4	4	4	2.5	
	10	5	3.5	4	3	
	15	4	2.5	4	2.5	
After Third Instillation	20-30	4	2.5	4	3	
	2	3	2	4	2.5	
	5	2	2	4	2.5	
	10	4	2	4	2	
	15	5	2.5	5	2.5	
	25	4	2.5	4	2	
	30	4	2.5	4	2.5	

20 minutes later widening of the lid and dilatation of the pupil began, the conjunctiva being greatly congested; undoubtedly this action was due to the effect of the horse serum. After the second and third instillations the course of events was more rapid and associated with general excitement. There was congestion of the lid, the conjunctiva, the iris and the ear lobe as early as 7 minutes after the first instillation; later there was congestion of the disk and the retinal vessels of the treated eye, renewed after each instillation, with hypersecretion of tears beginning 22 minutes after the first instillation. At the same time there were dyspnea, excitement, and increased reflex action. After death there was marked congestion of the central nervous system with many small points of hemorrhage, also congestion of the mucous membranes; there was a hemorrhagic spot on the upper part of Tenon's capsule and hemorrhages about the entrance of the short ciliary arteries and the retinal vessels; there were also many subpleural hemorrhages, with congestion of the liver and other abdominal organs except the kidneys. After intraperitoneal injection of 6 cc of heated horse serum the congestion and edema of the lid and conjunctiva and the congestion of the iris and fundus, as well as the hypersecretion of tears, increased; there was also dilatation of the lid fissure and the pupil, but these were perhaps due more to the generous nervous stimulation.

TABLE 5

LID AND PUPIL REACTION OF SENSITIZED GUINEA-PIG TO INSTILLATION OF HEATED HORSE SERUM IN EYE

Time in Minutes	Left Eye (Instilled)		Right Eye (Control)		Remarks
	Lid Fissure in Mm.	Pupil in Mm.	Lid Fissure in Mm.	Pupli in Mm.	
Before	4	2.5	4	2.5	
After First Instillation	2	3.5	2	3.5	Left eye and earlobes congested. Left external congestion, chemosis and epiphora; iris hyperemia and congestion of optic disk
	3	4	2	3.5	
	7	3	2	3.5	
	11	6.5	4.5	6	
	15	4	3.5	4	
	20-22	4	3	3	
	24	5	3	4	
	30	4	2	4	
	40	2.5	2	3	
	50	3	2.5	4	
	60	3.5	3	4	
After Second Instillation	3	3	2	3.5	
	10	3	2	3	
	15	4.5	3.5	4	
	25	3	2.5	3	
	30	4	2.5	4	
After Third Instillation	3	3	2.5	3	Nose scratching
	7	2.5	2	3	
	10	6	2.5	6	
	15	4	3	4	
	20	2.5	2.5	2	
	30	2.5	2	3	

The Effect of Intraorbital Injection of Salt Solution Into Normal Guinea-Pigs.—Two-tenths of 2 cc of 0.9% salt solution was injected into the tissues of the left orbit of a female albino guinea-pig (table 6). This resulted in some contraction of the lids of both eyes and dilatation of the pupil of the injected eye occurring about half a minute after the injection; otherwise there were no special changes except a slight conjunctival injection and a little excess of tear

secretion. It would seem that the injection of this amount of salt solution into the orbit does not produce any special changes or reaction if made skilfully.

The Injection of Unheated Normal Horse Serum Into the Orbit of the Normal Guinea-Pig.—Two-tenths cc of unheated horse serum was injected carefully into the left orbit of a male albino. Immediately after the injection there were a slight contraction of the eyelids and moderate mydriasis of the left eye, followed by widening of the lids and an increased dilatation of the pupil lasting for one and a half hours and undoubtedly due to the stimulation of the serum of the corresponding smooth muscles. Within 3 minutes after the injection there was congestion of the injected eye with swelling of the lid and conjunctiva and increased lacrimation, these phenomena reaching their climax in 10 to 20 minutes, when the lids became everted, the discharge of tears very copious and congestion of the eyelids noted. About one hour after the injection there were only traces of inflammatory symptoms. It is interesting to note that the horse serum had a stimulating action on both lacrimal and harderian glands, due in some degree no doubt to the hyperemia.

TABLE 6
RESULTS OF INJECTION OF NORMAL HORSE SERUM INTO ORBIT IN NORMAL GUINEA-PIG

Time in Minutes	Lid and Pupil, Widths in Mm.		Remarks
	Left Eye	Right Eye (Control)	
Before injection	6.5 - 2.5	6.5 - 2.5	
After injection			
0.5	6 - 4	6 - 3.5	
1.5	6 - 3	6 - 2.5	Left eye:
3	6 - 2.5	5 - 2.5	Slight pericorneal injection
5	5 - 3	5 - 2.5	Conjunctival congestion, scratching of nose, tremors
7	5.5 - 3	5 - 2.5	Conjunctival discharge
10	7 - 3.5	6 - 3	Eversion of lid, chemosis
15-25	6.5 - 3	5 - 3	
30	6 - 3	5 - 2.5	Changes in left eye diminishing, fundus normal
35	6 - 3.5	5 - 2.5	
40	5.5 - 3.5	5 - 2.5	
50	7 - 3.5	5 - 2.5	
60	7 - 3	4.5 - 2.5	
70	6 - 3	5 - 2.5	

The Injection of Heated Normal Horse Serum Into the Orbit of the Normal Guinea-Pig.—Two-tenths cc of normal horse serum, heated to 56 C. for 30 minutes, was injected into the left orbit of a female albino (table 7). In about 11 minutes the width of the lid and pupil of the injected eye increased; after about one hour and a half gradual contraction set in. About 8 minutes after the injection, congestion and swelling of the conjunctiva and lids of the injected eye began; this increased and there were hypersecretion of lacrimal and harderian glands and some protrusion of the eyeball with congestion of the iris, these symptoms being most marked between 25 and 35 minutes after the injection.

The Injection of Normal Horse Serum Into the Orbit of a Sensitized Guinea-Pig.—A male, albino guinea-pig, received 2.5 cc of normal horse serum subcutaneously, and about 3½ months later 0.2 cc of heated horse serum was injected into the left orbit (table 8). There was an immediate closure of the lids and mydriasis caused by reflex action and then widening of the lid and stronger

mydriasis developed, the latter persisting for about 30 minutes, more marked than in the control nonsensitized animal. Similar changes developed about 10 minutes later in the other eye. The congestion and edema were also more marked in this animal than in the control animal. There was a greater hypersecretion of lacrimal and harderian glands in this animal than in the control. There was also marked congestion with hemorrhages of the retinal vessels of the injected eye, more marked than in the fellow eye. There seems to be no doubt that the smooth muscles, blood vessels and eye glands react more markedly to horse serum in the case of the sensitized guinea-pig. After death congestion and hemorrhages of some of the internal organs were discovered, especially in the brain and the retina; there was a noticeably vigorous peristalsis of the intestines.

TABLE 7

RESULTS OF INJECTION OF HEATED HORSE SERUM INTO ORBIT OF NORMAL GUINEA-PIG

Time in Minutes	Lid and Pupil, Widths in Mm.		Remarks
	Left Eye	Right Eye (Control)	
Before injection	5.5 - 3	5.5 - 3	
After injection			
1.5	7 - 4	7 - 4	
2	5.5 - 4	5.5 - 3	
3	5 - 3.5	5 - 2.5	
4	4 - 3	4 - 3	Left eye:
5	3.5 - 2.5	4 - 3	
7	4 - 3	4 - 3	Conjunctival congestion
11	4.5 - 3.5	4 - 3	
15	5 - 3.5	4 - 3	Both lids swollen, chemosis
17	5.5 - 3.75	4 - 3	
20	6 - 4	4 - 3	Marked epiphora
30	7 - 4	5 - 3.5	Changes in eye marked; fundus about normal
40	6.5 - 4	4 - 3	
50	7 - 4	5.5 - 4	
60	7 - 3.75	5.5 - 4	Iris hyperemia
70	6 - 4	5.5 - 4	
80	6 - 4	5.5 - 4	
90	5 - 3.5	4 - 3	Changes in left eye diminishing

At this point the results of some observations on the effect of chloroform may be mentioned briefly: In the beginning of chloroform narcosis of the guinea-pig the lids are closed, but when narcosis is established to excess the lids and pupils become widely dilated; if the narcosis is continued until the animal dies this dilatation persists for some time after death. The only changes observable in the internal organs is a moderate congestion, no hemorrhages being present.

The Effect of Subcutaneous Injection of Salt Solution and Normal Horse Serum in Normal and Sensitized Guinea-Pigs.—The injection of 2.5 cc of 0.9% salt solution subcutaneously on the back was followed in about 10 minutes by a slight narrowing of the lid fissure and of the pupil, the narrowing of the lid fissure increasing a little in the course of the next 30 minutes. These changes were not any greater than those in the control animal handled in the same way but not injected with salt solution. The injection of the same amount of salt solution into a guinea-pig that had received 0.01 cc of normal horse serum intraperitoneally one month before, was followed by an immediate widening of the lid fissure followed by contraction while the pupils remained unchanged; there were no vascular or secretory disturbances.

TABLE 8

RESULTS OF INJECTION OF NORMAL HORSE SERUM INTO ORBIT OF SENSITIZED GUINEA-PIG

Time in Minutes	Lid and Pupil, Widths in Mm.		Remarks
	Left Eye	Right Eye (Control)	
Before injection	6 - 2.5	6 - 2.5	
After injection			
2	4 - 3	4 - 3	
4	5 - 3.5	4 - 3	Pericorneal injection begins at left eye
5	5 - 2.5	4 - 3	Left eyelids and conjunctiva begin to swell; sneezing and weeping
6	5 - 4	4 - 3	
7	5 - 4	5 - 4	Left conjunctival congestion increasing; ear lobes congested; violent muscular movements
8	5 - 3.5	4 - 3	
10	5 - 4	5 - 4	Both eyes and ears congested
13	5 - 4	5 - 4	Left iris and fundus congested strongly
15	5 - 4	5 - 4	Marked pericorneal injection and whitish discharge
20	5 - 3.5	5 - 3.5	Dyspnea
25	5 - 4	5 - 4	Retinal hemorrhages at left eye
30	5.5 - 3.5	5 - 3	Chloroform inhalation
40	Slow respiration, unconsciousness
60	
180	5.5 - 5	5.5 - 4.5	

TABLE 9

RESULTS OF SUBCUTANEOUS INJECTION OF NORMAL HORSE SERUM IN SENSITIZED GUINEA-PIGS

Time in Minutes	Lid and Pupil, Widths in Mm.		Remarks
	Left Eye	Right Eye (Control)	
(Before)	6.5 - 3.5	6.5 - 3.5	
3	6 - 4	6 - 4	Scratching of nose
5	6 - 4	6 - 4	Symptoms of asphyxia
7	5 - 4	5 - 4	
10	5 - 3.75	5 - 3.75	Congestion of conjunctiva, ears, and nose
15	4.5 - 3	4.5 - 3	Epiphora marked, iris hyperemic
20	4.5 - 3	4.5 - 3	Fundus congested
22	4 - 2	4 - 2	
25-30	4 - 2.5	4 - 2.5	
32-40	4 - 3	4 - 3	
42	3 - 2	3 - 2	
45	3 - 2.5	3 - 2.5	
47-48	3 - 2	3 - 2	
49-50	4 - 2	4 - 2	
51	2.5 - 2	2.5 - 2	
52-55	3 - 2	3 - 2	
57-71	3 - 2.5	3 - 2.5	
75	2 - 2.5	2 - 2.5	
77	3 - 2.5	3 - 2.5	
80	2.5 - 2.5	2.5 - 2.5	
82	4.5 - 2.5	4.5 - 2.5	
85	4 - 3	4 - 3	
90	3 - 2.5	3 - 2.5	
97	3.5 - 2	3.5 - 2	
100	3 - 2	3 - 2	
105	4 - 3.5	4 - 3.5	
110	3 - 3	3 - 3	
115	3 - 2.5	3 - 2.5	
120	3.5 - 2.5	3.5 - 2.5	
150	4 - 3.5	4 - 3.5	

The injection of 2.5 cc normal horse serum subcutaneously on the back into albino guinea-pigs did not cause any changes in the condition of the lid fissure or pupil until about 5 minutes later when there was contraction of both lasting for over 2½ hours; at the same time there was some congestion of the conjunctiva, ear lobes, nose and nails. In about 10 minutes tears began to flow; hypersecretion lasted about as long as the congestion, namely, 50 minutes. The fundus showed congestion 30 minutes after the injection. There was some increase in the frequency of respiration and also in the rectal temperature. This shows that normal horse serum subcutaneously in the dose of 2.5 cc, causes definite changes in the eyes—contraction of the lid fissure and pupil, and congestion in the fundus.

A female albino guinea-pig was injected intraperitoneally with 0.01 cc of normal horse serum and 26 days later 2.5 cc of normal horse serum were injected subcutaneously (table 9); respiratory difficulty and general nervous symptoms soon followed; there was some eversion of the lids and marked mydriasis, lasting for about 10 minutes, whereupon an increasing contraction of the lids and pupils developed with congestion of the conjunctiva, lacrimation, and congestion of the iris and fundus. There was a moderate fall in the rectal temperature. The eversion of the lids noted in this experiment, the marked mydriasis, the primary lid contraction, and mydriasis followed by narrowing of lid and pupil would appear to be definite anaphylactic eye phenomena.

TABLE 10
RESULTS OF INTRAVENOUS INJECTION OF HORSE SERUM INTO NORMAL GUINEA-PIGS

Time in Minutes	Lid and Pupil, Widths in Mm.			
	Injected Guinea-Pig		Control Guinea-Pig	
	Right Eye	Left Eye	Right Eye	Left Eye
(Before)	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75
10
15	4.5 - 3.75	4.5 - 3.75	5.5 - 3.75	5.5 - 3.75
30	4.5 - 3.75	4.5 - 3.75	5.5 - 3.75	5.5 - 3.75
40	4 - 3.25	4 - 3.25	5.5 - 3.75	5.5 - 3.75
50	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75
60	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75
90	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75
120	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75	5.5 - 3.75

The Effect of Intravenous Injection of Salt Solution in Normal and Sensitized Guinea-Pigs.—The intrajugular injection of 2.5 cc of a 0.9% salt solution was followed by a slight narrowing of the lid fissures and contraction of the pupil within 30 minutes, as compared with the control animal; there was also some diminution in the respiratory frequency and the rectal temperature in the injected animals, but there were no changes in the interior of the eye. Practically the identical changes took place in a guinea-pig previously sensitized with horse serum and then injected with 2.5 cc of salt solution.

Intravenous Injection of Normal Horse Serum Into Normal and Sensitized Guinea-Pigs.—The intravenous injection of 2.5 cc of normal horse serum into a normal guinea-pig was followed by a moderate contraction of the lids and pupil reaching its maximum about 40 minutes after the injection, slight congestion of the conjunctiva, iris, and fundus with lacrimation. Though some lessening in respiratory frequency and in the rectal temperature was noticed soon after the injection, it passed away before long (table 10).

A female, pigmented guinea-pig was injected intraperitoneally with 0.01 c c of normal horse serum and about 40 days later 2.5 c c of normal horse serum were injected into the jugular vein (table 11). In this case a typical anaphylactic death with convulsions took place 4 minutes after the second injection, associated with a marked mydriasis and widening of the lids but followed in 6 minutes after death by contraction, reaching maximum in 40 minutes. In a second similar experiment, in which 2 c c of normal horse serum were injected intravenously into an animal sensitized in the same way, the results were practically the same, postmortem myosis beginning in 6 minutes and reaching maximum in one hour.

TABLE 11
EYE PHENOMENA IN SUDDEN ANAPHYLACTIC DEATH

Time in Minutes	Lid and Pupil, Widths in Mm.					
	1			2		
	Right Eye	Left Eye	Remarks	Right Eye	Left Eye	Remarks
(Before)	6 - 3.75	6 - 3.75	Convulsions Optic disk pale; death	6 - 3	6 - 3	Convulsions Optic disk pale; death
2	6 - 3.75	6 - 3.75		6.5 - 4	6.5 - 4	
4	6 - 4.5	6 - 4.5		6.5 - 4	6.5 - 4	
After death			Optic disks pale			Optic disks pale
1	6.5 - 5	6.5 - 5		
6	6 - 3.5	6 - 3.5		5.5 - 3	5.5 - 3	
11	5.5 - 2.5	5.5 - 2.5				
13		5.5 - 2	5.5 - 2	
18		5.5 - 2	5.5 - 2	
26	5.5 - 2.25	5.5 - 2.25		4.5 - 2	4.5 - 2	
36	4.5 - 2.25	5 - 2		4.5 - 2	4.5 - 2	
46	4 - 1.75	5 - 1.5		4.5 - 2.25	4.5 - 2	
60	4 - 1.75	4 - 1.5		4.5 - 2	4.5 - 1.5	
105		3.5 - 1.75	3.5 - 1.5	
120	5 - 1.75	4 - 1.5		
225		3 - 1.75	3 - 1.75	
300		2 - 1.75	2 - 1.75	

TABLE 12
EYE PHENOMENA IN ACUTE ANAPHYLACTIC SHOCK

Time in Minutes	Lid and Pupil, Widths in Mm.		Remarks
	Right Eye	Left Eye	
(Before)	4.75- 3	4.15- 3	Convulsions
5	5.5 - 5	5.5 - 5	
1	5.5 - 5	5.5 - 5	
13	5 - 5	5 - 5	
15	4 - 5	4 - 5	Congestion of conjunctiva and eye in general. Some relaxation Lacrimation
20	4 - 5	4 - 5	
30	4 - 5	4 - 5	
35	4 - 5	4 - 5	Muscular power returning
40	4 - 2	4 - 2	
50	4 - 3.5	4 - 3.5	
60	3 - 3	3 - 3	
80	3 - 4	3 - 4	
After death from chloroform:			
2	2 - 5	2 - 5	
5	4 - 4	4 - 4	
10	4 - 5	4 - 5	

In the case of a guinea-pig injected 4 months before with diphtheria toxin-antitoxin, the intrajugular injection of 2.5 c.c. of normal horse serum was followed by an acute but nonfatal typical anaphylactic shock with characteristic respiratory symptoms, loss of reflexes, fall of rectal temperature, etc., and the following notable eye phenomena: beginning almost immediately after the injection a marked dilatation of the lid fissure and pupil of both eyes was noted, the lid fissures beginning to contract in 15 minutes and the pupils in 40. Conjunctival congestion, lacrimation, and hyperemia of the iris and fundus developed. The widening of the lid fissures and pupils might have been the result in part of the accompanying asphyxia, and control experiments were made (table 12): A male, albino guinea-pig was choked rapidly by pressure on isolated windpipe, immediately after the intravenous injection of 2.5 c.c. of normal horse serum, and a normal female pigmented guinea-pig choked similarly but slowly without any injection. In both instances, the lid dilated and strong mydriasis developed and persisted long after death (table 13).

TABLE 13
THE RESULTS OF MECHANICAL ASPHYXIA IN NORMAL GUINEA-PIGS

Time in Minutes	Lid and Pupil, Widths in Mm.					
	Normal Guinea-Pig			Injected Intravenously with Horse Serum		
	Right Eye	Left Eye	Remarks	Right Eye	Left Eye	Remarks
(Before)	6 - 4	6 - 4		6.5 - 3.5	6.5 - 3.5	Daylight
(Before)		6 - 3.75	6 - 3.75	Electric light
2	6 - 5.5	6 - 5.5		6.5 - 4	6.5 - 4	
4	No congestion of the eyes
5	6 - 5.5	6 - 5.5	Retina congested			
15	No congestion at any time of conjunctiva			
21	5 - 5.5	5 - 5.5	Retinal conges- tion marked			
22	5 - 5.5	5 - 5.5				Retina pale
After death		6.5 - 4	6.5 - 4	
3	4 - 5.5	4 - 5.5		5.5 - 4	5.5 - 4	
5		5.5 - 5.5	5.5 - 5.5	
6	5 - 5.5	5 - 5.5		5.5 - 5.5	5.5 - 5.5	
8		5 - 5.5	5 - 5.5	
12	5 - 5.5	5 - 5.5		5 - 5.5	5 - 5.5	
18		4.5 - 5.5	4.5 - 5.5	
26	5 - 5.5	5 - 5.5		4 - 5.5	4 - 5.5	
28				
36				
38				
60				
120				

When the postmortal states of the lids and pupils in both experiments of anaphylactic sudden deaths (tables 11 and 12) are compared with the following mechanical asphyxia, a strong myosis (1.5 mm. in 46 minutes to 1 hour) seems to be a characteristic feature of anaphylactic death.

Mydriatics and myotics have no influence on the postmortal state of the pupil, causing no characteristic effects usually, adrenalin

being the only exception to this general rule. V. Szily and Arisawa⁴ observed a pupil contraction in the rabbit after previous injection with fowl or sheep serum into vitreous chamber and 4 weeks later intravenous injection of 5 c c of homologous serum. The pupil contraction was brief and limited to the treated eye only. They give no explanation of the mechanisms. Woods⁵ noted contraction of the pupil in the dog when previously sensitized with uveal tissue and later perfused with oxygenated normal defibrinated dog serum containing that specific antigen. Neither author seems to have observed any anaphylactic lid phenomenon or primary dilatation of the pupil as part of the reactions of anaphylaxis.

According to Schultz's¹ work on excised blood vessels, it would be expected that a vasoconstrictive action would result in anaphylaxis, but my experiments indicate, on the contrary, a vasodilating action on the tissues of the eye and adnexa, on the earlobes, nose and nails. Comparison of the lesions of anaphylactic shock and of pure mechanical asphyxia indicates that the well-known visceral congestion and hemorrhages in these conditions are due both to indirect asphyxial and direct anaphylactic action on blood vessels. This affirmation of an anaphylactic vasodilating action is supported by Frölich's⁶ experiments on the peritoneum of sensitized frogs.

SUMMARY AND CONCLUSIONS

Generally speaking, the immediate effect of the application of horse serum applied to the eye of normal and of sensitized guinea-pigs is dilatation of the lid and pupil succeeded by contraction. In the sensitized animal, however, the response is more prompt and vigorous.

The primary dilatation would seem to be the result of stimulation of the tarsal smooth muscles of the lid and the dilator smooth muscle of the iris. In cases of anaphylactic shock the eye phenomena mentioned may be increased as a result of the asphyxia and other general effects.

The secondary narrowing of the lid fissure and contraction of the pupil may be explained as due to the loss of the tonus of the tarsal muscle and of the dilator pupillae on the one hand and to the contraction of the sphincter muscle of the iris associated with congestion on the other hand. The primary stimulation of the tarsal and dilator muscles

⁴ Die Anaphylaxie in d. Augenheilk., 1914, pp. 139 and 149.

⁵ Studies of Musser Dept. of Research Med., 1916-1918, 4, pp. 13 and 14

⁶ Ztschr. f. Immunitätsf., 1914, 20, p. 476.

may result from very small doses of the active principle concerned and may pass easily into a state of exhaustion from overstimulation. The stimulation of the sphincter of the pupil requires a stronger action. The primary and secondary effects on the smooth muscles of the lid and iris of guinea-pigs suggest that the anaphylactic action involves the ends of both the true and parasympathetic nerve fibers on both sets of plain muscles in the lid and the iris.

In addition to these phenomena, anaphylactic intoxication with horse serum may be associated with more or less well marked circulatory disturbances with edema and congestion of lid, conjunctiva, iris and ocular fundus, hemorrhages of epibulbar and retinal vessels. The direct application to the eye of horse serum, heated or unheated, may cause vascular dilatation in the normal, but hemorrhage is observed, especially in the sensitized guinea-pigs, and this effect may be obtained independently of asphyxia; hence it is due to direct action on the vessels.

In the normal and sensitized guinea-pig horse serum may cause hypersecretion of the lacrimal and harderian glands, especially on local application in the sensitized animal; this effect may be the result of the hyperemia, but direct stimulation of the glands and their nerves is not excluded.

In addition to the characteristic postmortem appearances of anaphylactic shock, seen especially in the dilatation of the lungs, congestion and hemorrhagic extravasation, we have also to make special note of a rapid and strong contraction of the pupil after anaphylactic death.

As all the anaphylactic eye phenomena—stimulation of plain muscles, circulatory disturbances and hypersecretion of eye glands—appear to be an intensification of the reaction that follows the application of horse serum to the normal eye, it may be assumed that the normal guinea-pig possesses small quantities of the antistances necessary for the anaphylactic reaction.

THE VIABILITY AND GROWTH OF *B. TYPHOSUS* IN BILE

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During the past twenty-five years there has been much discussion regarding the effects of bile and bile salts on members of the colon-typhoid group of organisms. Some workers suggest that the fluid is germicidal toward these forms, others think that it is not inhibitive and a third group believes that it is unfavorable at first and that later the organisms most fit and which have survived its influence propagate slowly.

Without discussion of the exact limitations of alteration of bile to render it most favorable to the growth of the organism or of the source from which it was taken for experimental purposes, it may be stated that the following authors believe that at least bile does not inhibit the proliferation of *B. typhosus* nor of *B. coli*: Von Fütterer,¹ Leubuscher,² Corrado,³ Fraenkel and Krause,⁴ Babes,⁵ Meyerstein,⁶ Hirokawa⁷ and Nichols.⁸ On the other hand, Mosse,⁹ Talma,¹⁰ Pies,¹¹ Fornet,¹² and Knick and Pringsheim¹³ believe that it is bacteriostatic. Dunschmann¹⁴ finds that sodium taurocholate favors the proliferation of *B. typhosus* while sodium glycocholate depresses it. The third point of view, namely, that bile inhibits slightly while the surviving forms grow slowly, has been held by Jordan,¹⁵ who states that bile hinders the growth of approximately one third of the viable cells of *B. coli*, and Ecker,¹⁶ who believes that many living cells of *B. typhosus* refuse to multiply in bile and that bile salts are distinctly unfavorable to it.

An interesting by-product relative to the effect of bile on intestinal bacteria is to be noted in the use of bile mediums for examination of water and milk for the detection of members of this group in sanitary analysis and also for

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¹ Münch. med. Wehnschr., 1888, 35, p. 315.

² Ztschr. f. klin. Med., 1890, 17, p. 472.

³ Atti della Accademia Medica de Roma, 1891.

⁴ Ztschr. f. Hyg. u. Infektionskr., 1889, 32, p. 97.

⁵ Berlin. klin. Wehnschr., 1899, 17, p. 362.

⁶ Centralbl. f. Bakteriöl., I, O., 1907, 44, p. 434.

⁷ Centralbl. f. Bakteriöl., I O., 1909, 53, p. 12.

⁸ Jour. Exper. Med., 1916, 24, p. 495.

⁹ Ztschr. f. klin. Med., 1899, 36, p. 527.

¹⁰ Centralbl. f. Bakteriöl., Ref., 1901, 29, p. 367.

¹¹ Arch. f. Hyg., 1907, 62, p. 107.

¹² Arch. f. Hyg., 1907, 60, p. 134.

¹³ Deut. Arch. klin. Med., 1911, 101, p. 137.

¹⁴ Ann. de l'Inst. Past., 1909, 23, p. 29.

¹⁵ Jour. Infect. Dis., 1913, 12, p. 326; Trans. 15th. Internat. Cong. Hyg., ii, p. 48.

¹⁶ Jour. Infect. Dis., 1918, 22, p. 95.

blood cultures in typhoid fever. Limbourg,¹⁷ Fraenkel and Krause,⁴ Grünbaum and Hume,¹⁸ Matzuschita,¹⁹ Jackson,²⁰ MacConkey,²¹ MacKonkey and Hill²² and Dunschmann¹⁴ have advocated the use of bile and bile salts for examination of *B. coli* and its near relatives. Such mediums are now being abandoned, however, following the reports unfavorable in character by Jordan,²⁵ Jordan, Russell and Zeit,²³ Longley and Baton,²⁴ Cumming,²⁵ Obst,²⁶ Tonney, Caldwell and Griffin.²⁷

Technic.—Experiments were attempted to learn (a) whether the strain of *B. typhosus* used in this work is viable in bile, and (b) whether it can multiply in such medium freely. The strain of *B. typhosus* used is that denominated as "No. 3" in this laboratory and was isolated originally from a carrier.

As opportunity allowed, there were obtained 7 samples of bile of which 5 were of human origin and one each from rabbit and beef. Those from human necropsy had the following histories: The first was from a case of carcinoma of the stomach; the second was from an old man with chronic passive congestion of the liver and no other distinctive lesions; the third was from a case of carcinoma of the pancreas with infection of the gallbladder. No information was obtained regarding the other two.

Of each sample 15 c c were placed in a tube and autoclaved for 15 minutes at 15 pounds' pressure since the material was not sterile when received. The rabbit bile was an exception in amount on account of its small volume, which was but 2 c c, and was put in an agglutination tube. When again cool, each was inoculated with two 2 mm. loopfuls of a 24-hour broth culture of *B. typhosus*. The cotton plugs were cut off even with the top of the tube and thrust down the bore sufficiently far so that a sterile rubber stopper could be placed in the mouth of the container to prevent evaporation during subsequent incubation. These preparations were then incubated at 37 C. with streaks made from them intermittently to determine whether inoculated organisms were still living. No attempt was made at first, therefore, to observe possible increase in numbers.

¹⁷ Ztschr. f. phy. Chem., 1889, 13, p. 196.

¹⁸ Brit. Med. Jour., 1902, 1, p. 1473.

¹⁹ Cent. f. Bakteriöl., Ref., 1902, 31, p. 505.

²⁰ Biol. Studies by Pupils of W. T. Sedgwick, 1906 p. 292.

²¹ Thompson-Yates Laboratory Reports, 1900, 3, p. 41; Jour. Hyg., 1908, 8, p. 322.

²² Thompson-Yates Laboratory Reports, 1901, 4, p. 151.

²³ Jour. Infect. Dis., 1904, 1, p. 641.

²⁴ Jour. Infect. Dis., 1907, 4, p. 397.

²⁵ Hyg. Lab. Bull., U. S. Pub. Health Serv., 1915, 104.

²⁶ Jour. Bacteriol., 1916, 1, p. 73.

²⁷ Jour. Infect. Dis., 1916, 18, p. 239.

At the termination of this experiment, streaks made from all of the cultures in bile were heavily positive for *B. typhosus*, and they were pure. Lack of time did not allow the experiment to continue a sufficient length of time for us to learn the period of possible viability of typhoid in the bile used. The results for each sample are given below.

TABLE 1
RESULTS OF EXPERIMENT

Number	Origin	Observation Period	Outcome
1	Human	160 days	Positive
2	Human	160 days	Positive
3	Rabbit	160 days	Positive
4	Beef	141 days	Positive
5	Human	126 days	Positive
6	Human	126 days	Positive
7	Human	126 days	Positive

Therefore, this strain of *B. typhosus* proved to be extremely long lived in human, ox and rabbit bile.

Next, knowledge concerning the rate of growth of *B. typhosus* in beef bile was sought with the following procedure: A stock of bile made up of the contents of four gallbladders was obtained from a local slaughter house and while still warm was placed in flasks in 100 c c quantities and autoclaved at 15 pounds' pressure for 15 minutes. It is interesting here to note the fact that this process of sterilization changed the hydrogen-ion concentration from P_H 7.2 in the raw bile to P_H 8.4 after cooking. Determinations were made according to the colorimetric method of Clark and Lubs.²⁸ A flask of bile was inoculated with 0.5 c c of a 24-hour broth culture of *B. typhosus* and plates poured as soon as possible after thorough shaking in order to estimate the initial number of organisms per c c. Dilutions were carried out according to the methods generally accepted for counting milk and water and 3 plates made of each. This flask was placed in the incubator at body temperature and left there during the course of the experiment. Thereafter, plate determinations with plain beef extract agar for the count per c c of the bile were carried out in $\frac{1}{2}$, 1, $1\frac{1}{2}$, 2, 3, 4, 6, 8, 24 and 48 hours. Most thorough agitation was given the test flask and dilution blanks before final plate inoculums were removed. Petri dishes thus poured were incubated at 37 C. for 24 hours in an inverted position and then counted.

²⁸ Jour. Bacteriol., 1917, 2, p. 1, p. 109 and p. 191.

TABLE 2
COUNTS OF SAMPLE OF BEEF BILE

Period	Count per C c
Immediate	155,000
½ hour	100,000
1 hour	70,000
1½ hours	67,500
2 hours	60,000
3 hours	47,500
4 hours	40,500
6 hours	43,000
8 hours	59,000
24 hours	292,000
48 hours	2,600,000

To serve as a partial check to this, beef broth was prepared, placed in 100 c c portions in Erlenmeyer flasks, sterilized in the same intensity and with reaction altered also to P_H 8.4. However, the broth was not used for 3 days, during which time the P_H had changed to 7.9, as may be expected to happen with any batch of medium. It was used without further alteration. Like technic was followed except that count determinations concluded with one at 12 hours when turbidity was becoming very evident.

TABLE 3
NUMBERS PER C C OF BEEF BOUILLON

Period	Count per C c
Immediate	200,000
½ hour	180,000
1 hour	217,500
1½ hours	210,000
2 hours	265,000
3 hours	575,000
4 hours	1,660,000
6 hours	26,400,000
8 hours	64,000,000
12 hours	187,500,000

It was realized that to be of greatest value, a check in beef broth should have a like reaction. Moreover, it was felt that possibly the lags were greater than they would be if both flasks were kept at or near 37 C. throughout the experiment. Therefore the series of count determinations was repeated with the broth on this second occasion in like P_H value to that of the bile which again was 8.4. The bile was taken from another lot of material collected at a different time and in both series was 48 hours old when manipulated. Also like the first, the second had been sterilized while still warm from the slaughter house. The flask of beef broth and that of bile had both been in the incubator over night and, except for the few moments while the initial

dilution was being prepared at each period, was kept there throughout the experiments. Unfortunately, a small number of the water flasks used in making dilutions proved to be contaminated because of imperfect sterilization—therefore the blanks which will be noted in table 4. The results in parallel are as herewith; figures are the quantity of organisms per c c.

TABLE 4
RESULTS OF SERIES OF COUNT DETERMINATIONS MADE WITH BROTH

	Time	Bile	Broth
Immediate	176,000
1/2 hour	140,000	190,000
1 hour	106,000	192,000
1 1/2 hours	133,000	297,000
2 hours	427,000
3 hours	1,160,000
4 hours	175,000	7,500,000
6 hours	530,000	48,500,000
8 hours	2,360,000	115,000,000
12 hours	3,975,000	430,000,000

It will be noted from these figures that *B. typhosus* in bile first passes through a period of germicidal action. The mortality in the first experiment during this phase amounted to nearly 74%, while in the second it approximated 40%. The second period is characterized by an exceedingly long lag and this in turn is followed by slow but progressive growth which, however, never increases to the point of production of turbidity. The outcome with *B. typhosus* is much like that of Jordan's¹⁵ with *B. coli* and the conclusions are in part identical with those of Ecker.¹⁶

The strain of *B. typhosus* which was originally isolated from a carrier is exceedingly long lived in human, beef and rabbit bile. Introduced into sterile beef bile, it is subjected to marked bactericidal activity on the part of the medium which brings about the death of a large fraction of the living forms. This is followed by an evident lag and in turn is succeeded by relatively slow but progressive proliferation.

FATE OF MICRO-ORGANISMS INTRODUCED INTO ISOLATED LOOPS OF THE INTESTINE *

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It is currently assumed that pathogenic organisms are killed or at least inhibited in their multiplication in the intestinal tract, mainly by the antagonistic action of the normal intestinal flora. This assumption is the basis for numerous suggested methods of bacteriotherapy which all have as their object the implantation of nonpathogenic antagonistic forms in the intestinal canal. We have attempted to test the validity of this fundamental assumption by studying the fate of micro-organisms introduced into isolated loops of the intestine.

Medium sized dogs were used. Under morphine-ether anesthesia, the abdomen was opened, and a ligature placed around the duodenum immediately below the pylorus. The duodenum was then flushed out with warm, physiologic salt solution introduced through a hypodermic needle inserted immediately below the ligature. During the washing-out process the duodenum was repeatedly milked downward to force out the solution. After the washing, a 20-30 cm. loop of the emptied duodenum was isolated between ligatures. This loop always included the entrances of the bile and pancreatic ducts. By the same method a 20-30 cm. jejunal loop was isolated.

Of the bacterial suspension, 5 cc were now injected into each loop by means of a hypodermic needle. The suspension usually contained from 5,000,000 to 150,000,000 organisms per cc. The contents of each loop were now thoroughly mixed by gentle massage, and a 3 cc sample of the mixed contents was removed for a study of bacterial antagonisms. This left approximately 2 cc of the original bacterial suspension in each loop.

The abdomen was then closed and the dog allowed to recover from the anesthetic. After a period, usually varying from 2 to 5 hours, occasionally as long as 12 hours, the animal was killed and the contents of each loop removed. The total number of residual specific micro-organisms in each loop was determined by the ordinary dilution method of milk and water analysis. Aerobic plates only were studied.

Data thus obtained from the use of 26 dogs are recorded in table 1.

Table 1 shows that *S. lutea* completely disappears from both loops of the intestine within 2 hours, and *B. violaceus* within 5 hours. *S. aureus* is greatly reduced in number in one loop within 3 hours and completely disappears from both loops within 12 hours. Even the spores of *B. subtilis* completely disappear from both loops within

that period of time. Red yeast disappears from one loop within 12 hours. *B. typhosus* is greatly reduced in number within 5 hours, but whether or not there is a complete disappearance of this micro-organism cannot be ascertained with the technic used.

TABLE 1

SPECIFIC ORGANISMS RECOVERED FROM ISOLATED LOOPS OF THE INTESTINE

The total number of bacteria injected into each loop usually varied from 10,000,000 to 300,000,000. In order to simplify the table, the number injected in each case has been recorded as 100%.

Micro-organism	Time in Hours	Percentage Recovered		
		Duodenal Loop, Percentage	Jejunal Loop, Percentage	Average Percentage
<i>S. lutea</i>	1½	0	50	25
	2	0	0	0
	4	0	0	0
	7	0	0	0
<i>B. violaceus</i>	2	180	30	105
	3	110	10	60
	5	0	0	0
<i>S. aureus</i>	3	130	30	80
	5	125	10	65
	7	15	5	10
	12	0	0	0
<i>B. subtilis</i> (contained spores)...	2	100*	100*	100
	12	0	0	0
<i>B. typhosus</i>	5	1†	1†	1
Red yeast.....	3	90	120	105
	5	185	130	155
	12	0	125	60
<i>Streptococcus</i>	3	50‡	105‡	80
	5	110‡	120‡	115
<i>B. anthracis</i> (contained spores)	3	100*	100*	100
	5	100*	100*	100
	12	70	140	105

* Approximate count.

† Total count of intestinal aerobes, mainly *B. coli*.

‡ Total count of hemolytic colonies.

The experiments with streptococci are inconclusive, due to the probable presence of other hemolytic organisms. *B. anthracis* spores are apparently not killed.

At the time of the necropsy, the duodenal loop always contained bile-stained fluid, the total fluid contents usually varying from 5 to 10 c c, occasionally being as great as 60 c c. The jejunal loop usually contained but from 1 to 2 c c of a slightly viscid fluid. This loop was usually washed out with salt solution to make the count.

Table 1 shows a more rapid disappearance of the micro-organisms from the jejunal loop in the majority of cases.

In order to test the rôle of bacterial antagonism in this disappearance, samples of the intestinal contents withdrawn from each loop immediately after the injection of the micro-organisms were incubated,

under both aerobic and anaerobic conditions. Counts of these incubated samples were made at the time of the necropsy. Data thus obtained are recorded in table 2.

TABLE 2
TEST OF BACTERIAL ANTAGONISMS

The original number of micro-organisms injected in each case is recorded as 100%.

Micro-organism	Time in Hours	Specific Micro-organisms in Incubated Controls		Other Aerobic Micro-organisms Present
		Aerobic, Percentage	Anaerobic, Percentage	
<i>S. lutea</i>	1½	120	150	1.5
	2	80	...	0
	4
	7	0	0	1000
<i>B. violaceus</i>	2	160	50	0.5
	3	105	70	5
	5	60	70	70
<i>S. aureus</i>	3	50	80	10
	5	85	85	...
	7	90	80	...
	12	1200	1800	1000
<i>B. subtilis</i> (contained spores)....	2	100*	100*	...
	12	75	65	50
<i>B. typhosus</i>	5
	3	80	80	1
Red yeast.....	5	125	100	1
	12	125	100	500
<i>Streptococcus</i>	3	105†
	5	160†	180	...
<i>B. anthracis</i> (contained spores)..	3	100*	100*	...
	5	100*	100*	...
	12	110	120	...

* Approximate counts.

† Total count of hemolytic colonies.

Table 2 shows little, if any, bacterial destruction in the incubated controls, except in the case of *S. lutea*. In certain of the longer incubations, e. g., *S. aureus*, there is even a considerable multiplication of the specific micro-organisms in spite of the presence of numerous presumable antagonistic forms. Multiplication is apparently inhibited with other micro-organisms, e. g., *B. violaceus* and *B. subtilis*; but whether or not this is due solely to bacterial antagonisms cannot be determined by the technic used.

CONCLUSIONS AND SUMMARY

Many specific micro-organisms injected into isolated loops of the intestine of dogs are destroyed in periods ranging from two to seven hours.

This destruction is not due to the antagonistic action of the normal intestinal flora.

There is evidently a distinct antibacterial mechanism in the intestine, the nature of which is at present unknown.

THE HEAT RESISTANCE OF SPORES WITH SPECIAL REFERENCE TO THE SPORES OF B. BOTULINUS

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This study is an investigation of the heat resistance of the spores of *B. botulinus* and the effects of several variable conditions which are ultimately responsible for their death. The conditions reported are: the effect of hydrogen and hydroxyl ion concentrations on thermal resistance and the effects of numbers of spores, desiccation, spore age and the presence of sodium chloride. A number of other factors and the intimate nature of the process of killing by heat under different conditions, and the laws governing it, are being studied. In addition to the purely academic aspects of the problem which this work covers, it is designed to furnish adequate criteria for the practical problems of sterilization and to insure the safety of processed foods with respect to botulism.

THERMAL DEATH RATE OF DIFFERENT STRAINS

For the purpose of our subsequent work and in order that the results obtained might have a practical application, it was necessary to determine the thermal resistance of the spores of all the available strains and to devote our attention to the most resistant one. There were sixteen strains in our laboratory at the time this work was begun, all of which were studied.

Strain 1. From the Museum of Natural History, New York; original source unknown; old laboratory strain of low virulence.

Strain 2. From the College of Physicians and Surgeons, New York; original source unknown; low virulence.

Strain 3. From San Jose, Calif. Isolated by Dickson from the crop of a chicken that died after eating home-canned string beans. The same beans had caused the death of one person. The strain was virulent.

Strain 4. From Hillsboro, Ore. Isolated by Dickson from the gizzard of a chicken that died after eating home-canned corn. The same corn had poisoned one person and about 50 chickens. The strain was virulent.

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Strain 5. From Escondido, Calif. It was isolated by Dickson from home-canned string beans from the same lot that had caused botulism in 7 persons, 4 of whom died. The strain was moderately virulent when first isolated.

Strain 6. Obtained from the Mulford Laboratories and supposed to have been isolated from cheese; low virulence.

Strain 7. From Seattle, Wash. Isolated by Dickson from the crop or gizzard of a chicken that had died after eating home-canned asparagus. The same asparagus had caused the death of four persons. Moderately virulent.

Strain 8. From Berkeley, Calif. Isolated by Dickson from a jar of home-canned string beans which was from the same lot that had poisoned a number of chickens. The strain was highly virulent.

Strain 9. Isolated by Dickson from dead chickens that were supposed to have eaten canned apricots; highly virulent.

Strain 10. Isolated by Dickson from hogs that were killed by eating canned peas; highly virulent.

Strain 11. Obtained from the New York Health Dept. Laboratories. Isolated by Nevin from a sample of home made cottage cheese; highly virulent.

Strain 12. Isolated by Graham and associated with forage poisoning in horses. The strain was toxic when first obtained in 1918; it has since lost its toxin producing power.

Strain 13. Isolated by Graham and associated with forage poisoning in horses; virulent.

Strain 14. Isolated by Graham from corn silage associated with an outbreak of forage poisoning among mules in Kentucky; low virulence.

Strain 15. Isolated by Edmondson at the Bureau of Chemistry, United States Dept. of Agriculture, from asparagus salad which had caused the death of four persons at Boise, Ida., January, 1919; virulent.

Strain 16. Isolated by Orr at the Harvard Medical School from ripe olives supposed to have caused botulism in 7 persons at Alliance, Ohio, in August, 1919; virulent.

The medium used for spore development was a mixture of two parts of finely ground sheep brain in one part of water. This was sterilized in the autoclave and used without adjustment. Its final reaction was neutral. The number and age of the spores was kept constant until the effect of variations in these factors could be determined and adequate controls applied. Spore development in the brain medium is rapid and reaches a total of approximately 15 million per c c when spore development is complete. This emulsion, undiluted, was exposed in 6 by three quarter inch test tubes for varying periods of time at 4 different temperatures in a DeKhotinsky bath. All the tubes were sealed with the exception of the one set exposed at 100 C. A loopful of the heated material was inoculated into meat infusion glucose agar tubes which were finally layered with paraffin oil and incubated at 37.5 C. The tubes were examined daily over a period of 4 months and the results recorded.

Spores approximately one month old were used. It was impossible to make all the exposures with spores of exactly the same age without introducing an error due to using spores from different bottles for the various exposures. As will be shown subsequently, the difference in thermal resistance due to a difference in age of several days when the spore is about one month old is too slight to be appreciable.

The four temperatures used were 100 C., 105 C., 120 C. and 95 C. for the less resistant strains. These, however, represent the thermometer reading inside the oil bath, and due to the radiation from the

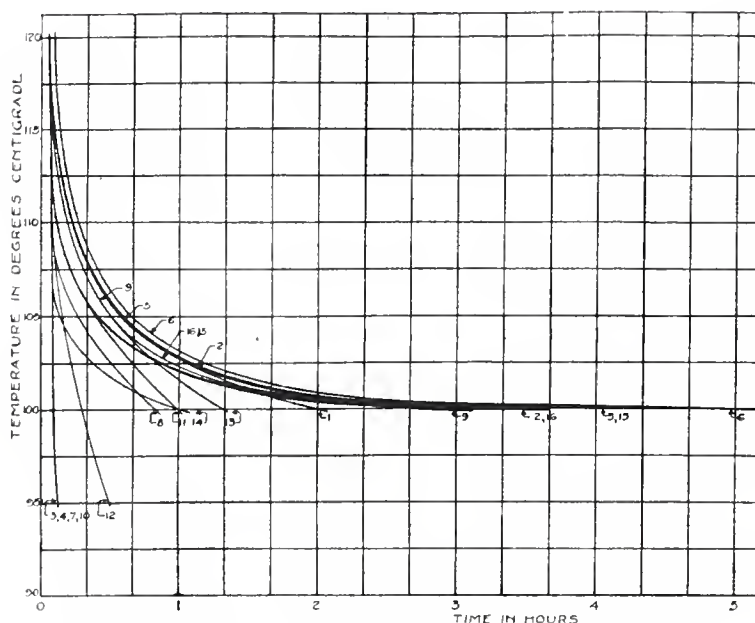


Chart 1.—Thermal death rate of spores of *Bacillus botulinus*.

exposed surface of the tubes is not the true temperature inside the tubes. The lag or period necessary to raise the inside of the tubes from room temperature to a constant temperature of 99 (with the bath at 100) was about 30 minutes. The lag in the case of the 105 degree exposure was 15 minutes and in that of the 120 degree exposure 8 minutes. It is evident from table 1 that in the latter case the organisms were destroyed during the period of lag when the temperature inside the tube was rising, and long before the bath temperature was reached.

Table 1 shows the results obtained using the 16 strains mentioned and chart 1 is a graphic representation of the same.

It is evident that under the best conditions for survival the most resistant spores of *B. botulinus* will be destroyed within 5 hours at 100 C., within 40 minutes at 105 C., and within 6 minutes at 120 C.

The thermal resistance varied considerably with the different strains. Spores, which were in apparently no other way dissimilar, showed marked differences in this respect, and the extent of the variation is well illustrated by the 100 C. exposure. At that temperature 4 strains were killed in 8 minutes, while it required from 3 to 5 hours at the same temperature to destroy the 6 that were most resistant. We have not attempted to correlate this variation with others that the strains may have exhibited. But it was evident that spore resistance is independent of toxin development, and that both toxic and atoxic strains may be either resistant or nonresistant. It was observed, however, that the most resistant strains sporulated most actively.

In a similar investigation, using 10 strains of *B. botulinus*, Burke¹ recently obtained results which are for the most part similar to our own. She, however, found one strain (X) which survived an autoclaving at 15 lbs. (121.3 C.) for 10 minutes and at 10 lbs. (115.6 C.) for 20 minutes. This same strain did not survive an autoclaving at 5 lbs. (108.8 C.) for 20 minutes. On the basis of this, Burke concludes that autoclaving at 15 lbs. (121.3 C.) for 10 minutes is insufficient to destroy the spores of *B. botulinus*. My results indicate that all spores of *B. botulinus* will be killed within 6 minutes at a bath temperature of 120 C. and that the greater number of them will be destroyed within 3 minutes. The results obtained by Burke may be explained by the fact that she used only pressure gage readings as an index of the temperature in the autoclave, and that there was apparently no adequate control of the true temperature inside the tubes.

EFFECT OF AGE AND NUMBER OF SPORES ON THE THERMAL DEATH RATE

In this part of the investigation and for all the subsequent work we used the spores of a single strain, 15, and varied one factor, keeping all the others constant. This strain was chosen from among the 16 strains because it best satisfied the conditions we applied. It came from an outbreak which was unquestionably botulism; it is, with the exception of strain 6, the most resistant; and it is an active gas producer on glucose mediums, forming spores readily and producing toxin.

Technic.—The technic was standardized and adhered to in all the various tests which follow. Bigelow and Esty² have described a technic somewhat similar to our own but differing from it in several

¹ Jour. Am. Med. Assn., 1919, 72, p. 88.

² Abstracts of Bact., 1920, 4, p. 10.

essential respects. Special tubes, 10 mm. inside diameter, 12 mm. outside diameter, and 12 inches long, were used. These were inoculated with the spore emulsion previously filtered through a sterile gauze filter to remove the large particles. The sides of the tubes were then thoroughly heated to within an inch of the top of the fluid. When these had cooled they were sealed in the flame and exposed in a de Khotinsky bath. A series was prepared in this manner and at the end of each interval of time one tube was removed, opened and glucose agar added, care being exercised to mix thoroughly the spore emulsion throughout the agar. If the spores had been in either acid or alkali, the tubes would be neutralized with equal parts of sterile and previously standardized hydrochloric acid or sodium hydroxide. Thus for each dilution of acid or alkali in which the spores were heated a sterile solution was used that would neutralize it in equal volume.

The glucose agar tubes were then layered with paraffin oil and incubated at 37.5 C. for at least 4 months.

There is evidently a gradual destruction of the spore, not an instantaneous killing (table 3). As the heating continues the spore appears to be gradually weakened. This is evident from the rate of development after exposure. As a general rule, the longer the period of exposure at any temperature, the longer the time required for the spore to vegetate until finally the injury is sufficient to destroy it. In one case growth did not appear until 91 days after exposure. This phase of the thermal death rate will be treated more fully in another paper.

In determining the effect of age on spore resistance several bottles of the same brain medium were inoculated at intervals from a 24-hour culture of *B. botulinus* and incubated. Four spore emulsions were used, respectively 34, 56, 122 and 163 days old. These were adjusted with 0.5% salt solution to 1,500,000 spores per c c, and tubes containing 1 c c were exposed at 98 C.

The 163-day spore survived for 30 minutes, but was killed in 60 minutes. The other 3 survived after 120 minutes heating, but were killed in 150 minutes. Of these, the 34-day spore showed the least retardation in vegetation, coming up on the fifth day. The 56-day old spore did not vegetate until the 28th day and the 122-day old spore did not vegetate until the 50th day.

TABLE 2
EFFECT OF AGE ON THERMAL RESISTANCE

Age of Spore in Days	Result of Heating at 98 C.	Retardation in Development of Last Surviving Spore
34	Survives 120 minutes; killed in 150 minutes	5 days
56	Survives 120 minutes; killed in 150 minutes	28 days
122	Survives 120 minutes; killed in 150 minutes	50 days
163	Survives 30 minutes; killed in 60 minutes	

It is evident from this that the oldest moist spore is the most severely injured during heating, and that the 34-day spore is the most resistant. This is contrary to the general conception that the resistance of a spore increases with age. This belief may have arisen from the fact that the aging of a spore is usually accompanied by drying, and it is this drying, not the aging, that is responsible. We have in progress investigations on the effect of drying on resistance to heat. This indicates that injury and death are the result of protein coagulation, and that the decrease of the moisture content of the spore decreases the effectiveness of the heating. If it were true that the resistance of a spore increased with age, logical reasoning would lead us to conclude that the spore cell is in itself immortal.

Another test was made combining both the effect of age and the effect of numbers of spores. Eighty and 18-day spores and concentrations of 1,500,000 and 15,000,000 per c c of each were used. The exposure temperature was 100 C. The results are given in table 3.

TABLE 3
EFFECT OF AGE AND DILUTION ON THERMAL RESISTANCE

	Exposure in Minutes at 100 C.							
	0	30	60	90	120	150	180	210
18-day spore, 15,000,000 per c c.....	1	2	4	9	16	30		
18-day spore, 1,500,000 per c c.....	1	4	5	17				
80-day spore, 15,000,000 per c c.....	1	5	29					
80 day spore, 1,500,000 per c c.....	1	5	7					

The numbers indicate the incubation period in days before growth appeared.

As in the former case, the younger spore was found to be more resistant in both suspensions.

Regarding the effect of numbers, it is evident in the case of the younger spores, both from the period of survival and the greater lag

in the more dilute emulsion at any one period, that the greater the concentration the greater the resistance. The theoretical consideration which this gives rise to will be offered in a later paper.

In the case of the 80-day spore it appears that dilution does not have such a pronounced effect. This indicates that as a culture ages there is a general retrogression, but that the more resistant individuals change more rapidly than the others with a resulting tendency toward stabilization, the ultimate resistance of the individual more nearly approximating the average resistance.

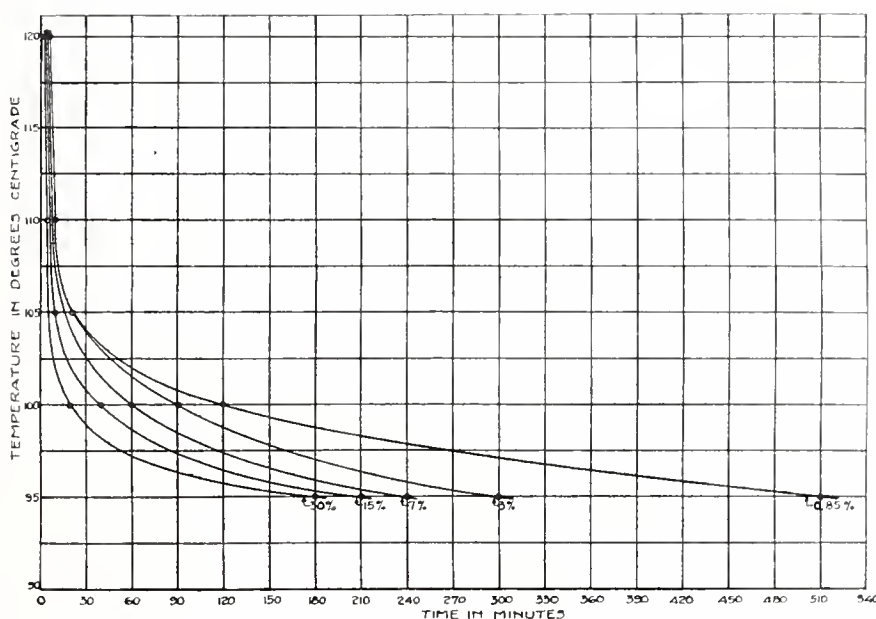


Chart 2.—Thermal death rate of spores of *Bacillus botulinus* in different concentrations of sodium chloride.

EFFECT OF SALT ON THERMAL DEATH RATE

The spores of strain 15 were exposed in 5 different strengths of sodium chloride at 5 different temperatures. The hydrogen-ion concentration was kept practically constant, that is, within limits that would have no practical effect. Ten parts of the salt solution were mixed with one part of the filtered spore emulsion and distributed so that each tube exposed contained 1,500,000 spores in 1.1 c c volume. In this, as in all other work in which dilutions were used, the molar strength was made the basis of comparison.

The results are given in table 4 and are represented graphically in chart 2.

TABLE 4
SODIUM CHLORIDE

Strength		+ [u]	pH	Exposure in Minutes																																
				At 95 C.												At 100 C.												At 105 C.			At 110 C.			At 120 C.		
In	Per			0	30	60	90	120	150	180	210	240	270	300	330	360	390	420	450	480	510	0	10	20	30	40	50	60	90	120	0	5	10	0	3	6
5 M	30		8.0×10^{-8}	7.1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2.5 M	15		4.5×10^{-8}	7.35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1.2 M	7		3.5×10^{-8}	7.46	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.5 M	3		4.6×10^{-8}	7.34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0.14 M	0.85		4.6×10^{-8}	7.34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates growth; — indicates no growth.

TABLE 5
HYDROCHLORIC ACID

Strength		+	pH	Exposure in Minutes																													
				At 95 C.												At 100 C.												At 105 C.		At 110 C.		At 120 C.	
				0	30	60	90	120	150	180	210	240	270	300	330	0	10	20	30	40	0	10	20	0	5	10	0	3	6				
In Molar Solution	Per Cent.																																
0.2 M	0.7	4.5×10^{-2}	1.35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
0.1 M	0.35	1.6×10^{-2}	1.80	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
0.05 M	0.18	1.5×10^{-3}	2.82	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
0.01 M	0.04	1.9×10^{-6}	5.72	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				

+ indicates growth; — indicates no growth.

The 5 temperatures of exposure used in this, as in all the subsequent work, were 95 C., 100 C., 105 C., 110 C. and 120 C. These represent the bath temperatures and are given in the tables. The liquid inside the tubes does not reach the bath temperature until several minutes after they had been exposed. The period necessary to raise the temperature will vary with the particular technic employed. In our case it was 8 to 12 minutes. It is evident from this, therefore, that at exposures of 120 C., in which killing occurs in 6 minutes or less, the actual thermal death point of the organism is several degrees lower.

To what particular factor or to what combination of factors the toxicity of sodium chloride is due, we are as yet unable to conclude. The various possibilities that present themselves are the action of the free sodium and chlorine ions, the undissociated salt, and the physical factors such as change in osmotic pressure and the presence of a large amount of electrolyte. It is also doubtful just what the mechanism of the action is, whether it is a direct intoxication in the sense of a chemical poisoning or what appears more probable, an increase in the coagulability of the cell protein.

EFFECT OF H AND OH ION ON THERMAL DEATH RATE

In determining the effect of the hydrogen and hydroxyl ion concentration on the thermal death rate, the technic previously described was used. Hydrochloric acid, oxalic acid, tartaric acid, lactic acid, acetic acid and citric acid were used. One alkali, sodium hydroxide, was also used. Each of these was made up in 4 dilutions on the basis of molar strength, and their effect was determined on measured numbers of the free spore at 5 different temperatures ranging from 95 C to 120 C.

Ten parts of the solution were mixed with one part of spore emulsion so that each tube contained 1,500,000 spores in 1.1 c c of volume. The hydrogen-ion concentration of the mixture was determined electrometrically. After exposure the tubes were neutralized and 10 c c of 1% glucose agar added. They were then layered with paraffin oil and incubated for at least 4 months at 37.5 C.

Tables 5 to 10 inclusive give the results obtained with 4 dilutions of each of the 6 acids previously mentioned. The dilutions of each acid were made to cover a hydrogen-ion range from a point close to neutrality to the practical limit of its dissociation with the spore emulsion present. Charts 3 to 8 inclusive are graphic representations of the same.

TABLE 6
OXALIC ACID

Strength		+	[n]	D _n	Exposure in Minutes																													
					At 95 C.												At 100 C.						At 105 C.						At 110 C.				At 120 C.	
					0	30	60	90	120	150	180	210	240	270	300	330	360	390	0	10	20	30	40	50	0	10	20	0	5	10	0	3	6	
0.1 M	1.3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
0.05 M	0.63	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
0.01 M	0.13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		
0.001 M	0.013	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		

+ indicates growth; — indicates no growth.

TABLE 7
TARTARIC ACID

Strength		Exposure in Minutes																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
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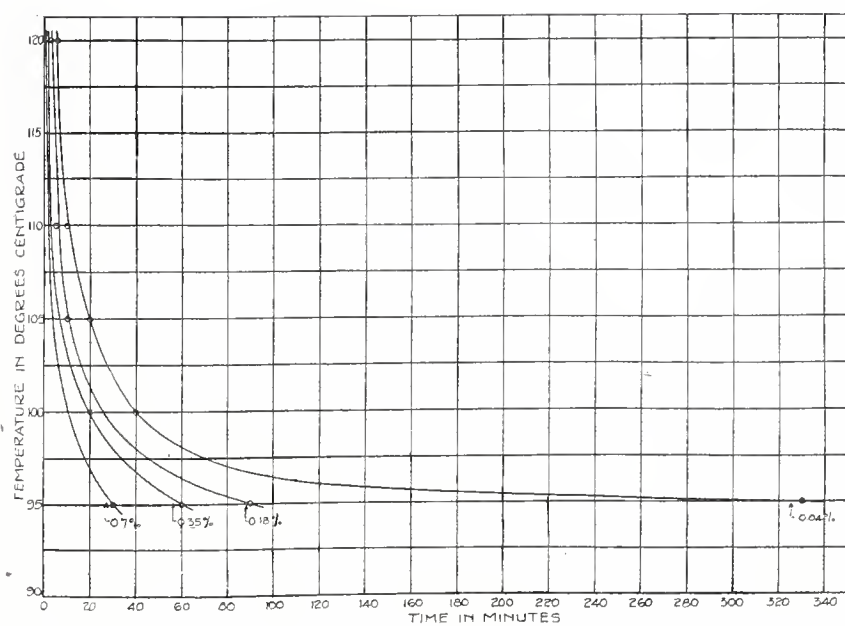


Chart 3.—Thermal death rate of spores of *Bacillus botulinus* in hydrochloric acid.

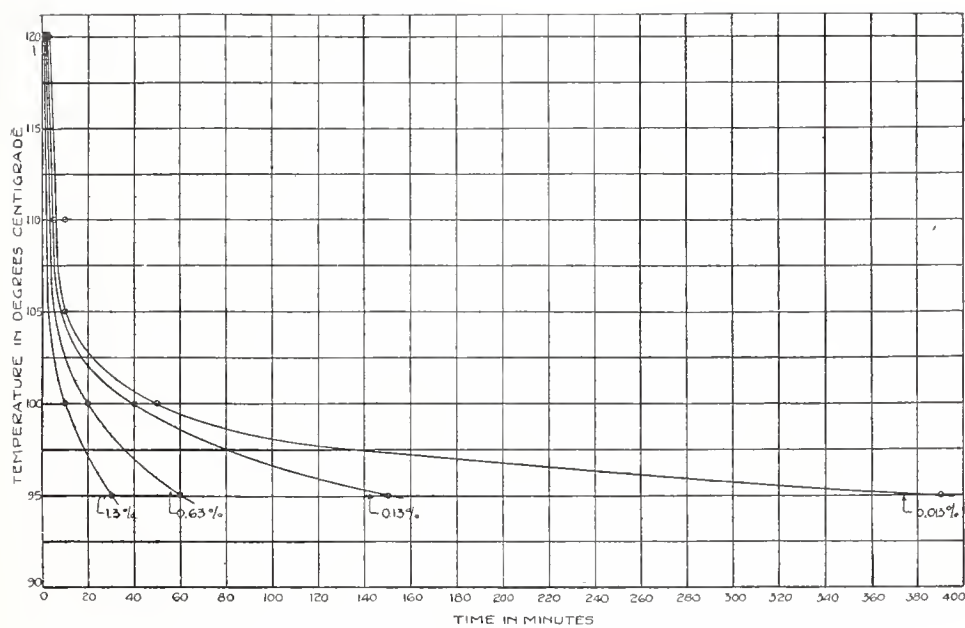


Chart 4.—Thermal death rate of spores of *Bacillus botulinus* in oxalic acid.

TABLE 8
LACTIC ACID

Strength		+ [n]	pH	Exposure in Minutes																				
In Molar Solution	Per Cent.			At 95 C.						At 100 C.						At 105 C.			At 110 C.			At 120 C.		
		0	30	60	90	120	150	180	210	240	0	10	20	30	40	0	10	20	0	5	10	0	3	6
0.2 M	1.8	+	+	—	—	—	—	—	—	—	+	+	—	—	—	+	+	—	+	+	—	+	—	—
0.1 M	3.46	+	+	+	—	—	—	—	—	—	+	+	—	—	—	+	+	—	+	+	—	+	—	—
0.05 M	3.59	+	+	+	+	—	+	—	—	—	+	+	+	+	—	+	+	—	+	+	—	+	—	—
0.01 M	5.00	+	+	+	+	+	+	+	+	—	+	+	+	+	—	+	+	—	+	+	—	+	—	—

+ indicates growth; — indicates no growth.

TABLE 9
ACETIC ACID

[illegible]

+ indicates growth; — indicates no growth.

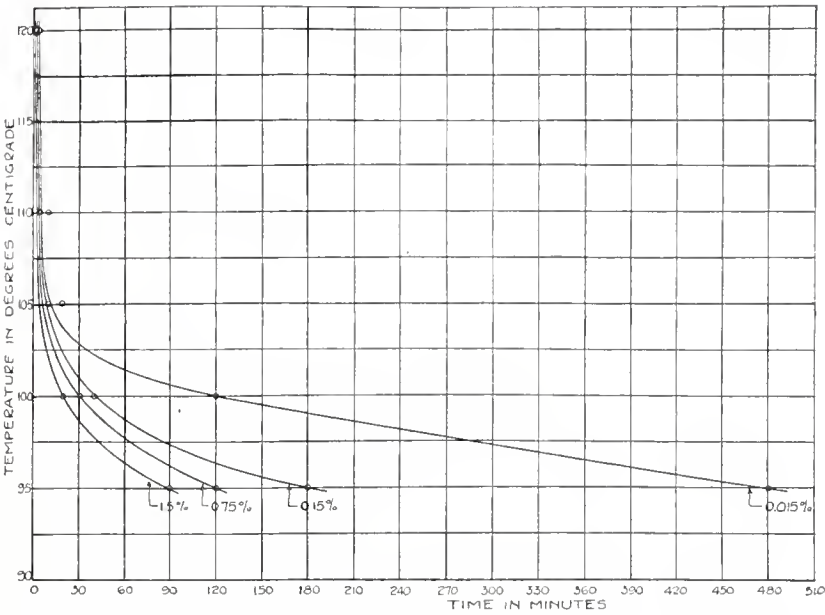


Chart 5.—Thermal death rate of spores of *Bacillus botulinus* in tartaric acid.

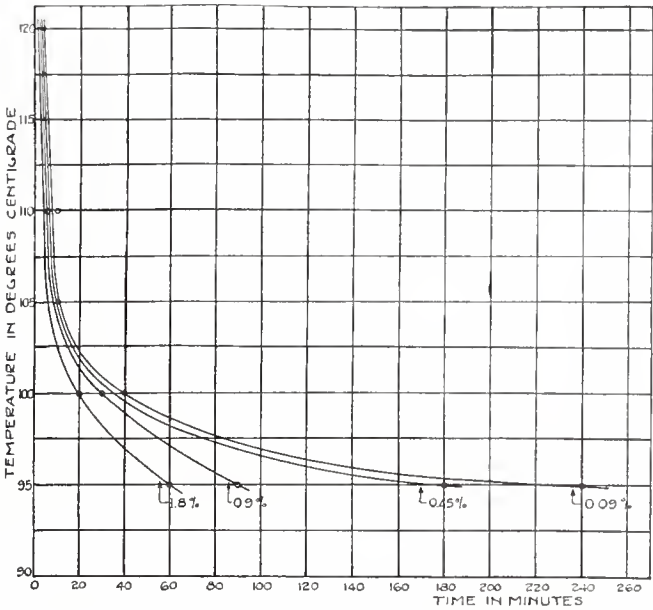


Chart 6.—Thermal death rate of spores of *Bacillus botulinus* in lactic acid.

TABLE 10
CITRIC ACID

Strength		+ [n]	pu	Exposure in Minutes																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
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In Molar Solution	Per Cent.			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates growth; — indicates no growth.

TABLE 11
SODIUM HYDROXIDE

Strength		+ [u]	pH	Exposure in Minutes																																						
				At 95 C.								At 100 C.								At 105 C.				At 110 C.				At 120 C.														
In Molar Solution	Per Cent.			0	30	60	90	120	150	180	210	240	270	300	330	360	390	420	450	480	0	10	20	30	40	50	60	0	10	20	30	40	50	60	0	5	10	0	3	6		
0.2 M	0.8	3.8×10^{-12}	11.42	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	+	+	+	—	—	—	—	—	—	—	+	+	+	—
0.1 M	0.4	7.5×10^{-11}	10.13	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—
0.05 M	0.2	5.5×10^{-10}	9.26	+	+	+	+	—	+	+	+	+	—	—	—	+	—	—	—	—	—	+	+	+	+	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—
0.01 M	0.04	6.0×10^{-9}	8.22	+	+	+	+	—	+	+	+	+	+	+	+	+	+	+	+	—	—	+	+	+	+	—	—	—	+	+	+	—	—	—	—	—	—	—	—	—	—	—

+ indicates growth; — indicates no growth.

Oxalic acid is the only acid in which the negative ion appeared to exert any toxic effect. In the very high dilution, in this case 0.001 molar, the emulsion is alkaline due to the fact that the spore emulsion with which the acid is mixed becomes alkaline during the growth of the organism. This change shall be taken up separately in another place.

Since for the practical purposes of our problem we were interested more particularly in the acid range, only one alkali was used to determine the effect of hydroxyl ion, and to cover the range of hydrogen-ion concentration from a maximum to a minimum. Four dilutions of sodium hydroxid, 0.2 molar, 0.1 molar, 0.05 molar and 0.01 molar were used. The results are shown in table 11 and are graphically represented in chart 11.

In order to determine the general effect of hydrogen ion on thermal death rate the thermal death points at one temperature were plotted with P_H as the abscissa and time necessary to produce death as the ordinate. Two such curves are given, one for a temperature of 95 C. (chart 10) and the other for 100 C. (chart 11). At the higher temperatures the killing is too rapid to make such curves of any value.

It is evident from the accompanying charts that the greatest resistance to killing by heat occurs close to the neutral point and that as the hydrogen-ion concentration increases it is accompanied by a rapid reduction in that resistance. It is, furthermore, apparent that the most pronounced change in resistance occurs just beyond the neutral point and that as the hydrogen-ion concentration increases further the decrease in resistance for a similar change in hydrogen-ion concentration becomes less. In other words, the rate of loss of resistance decreases as the hydrogen-ion concentration increases.

Comparing the two charts, we can also conclude that this change in rate with change in hydrogen-ion concentration becomes more pronounced as the temperature increases.

Regarding the alkaline side of the curve, it appears that the hydroxyl ion has nearly, if not exactly, the same effectiveness as the hydrogen ion.

PRACTICAL APPLICATION

In order that the practical application of these results might be facilitated charts 12 and 13 were prepared. Chart 12 gives the thermal death point for the spores of *B. botulinus* under the conditions of our

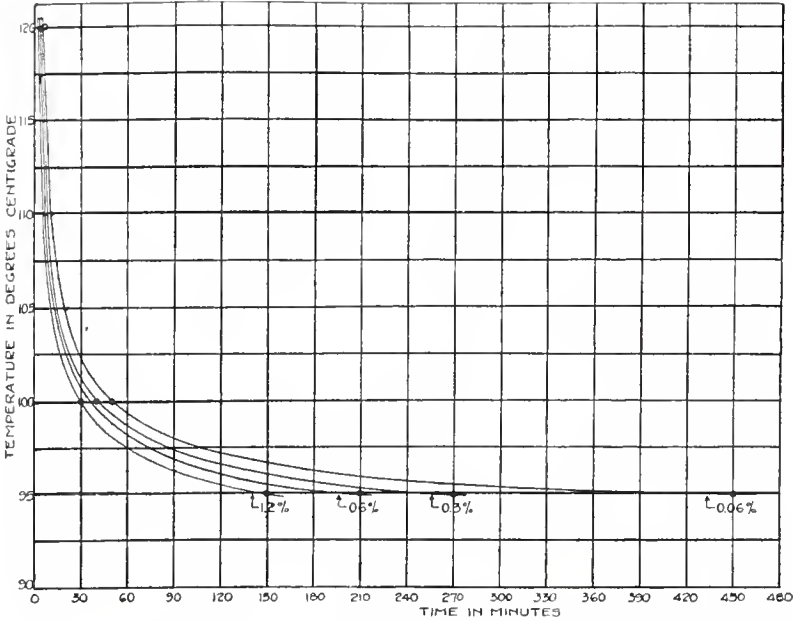


Chart 7.—Thermal death rate of spores of *Bacillus botulinus* in acetic acid.

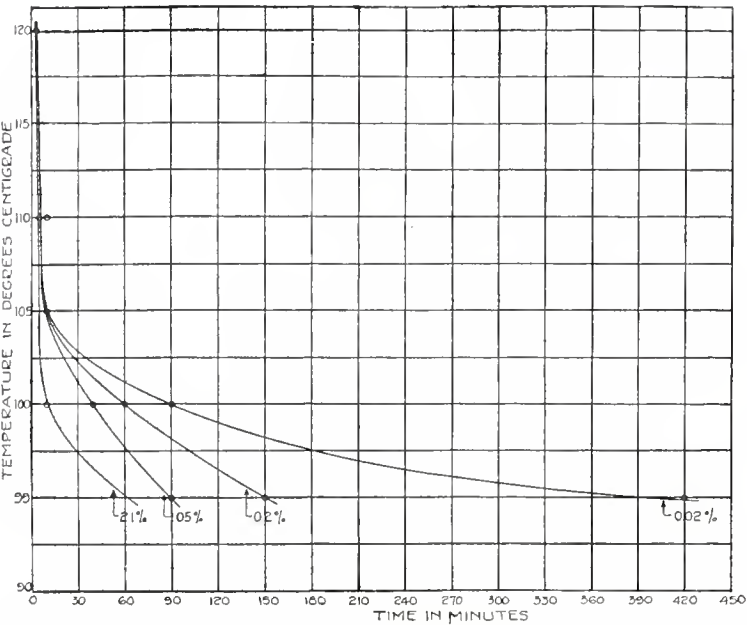


Chart 8.—Thermal death rate of spores of *Bacillus botulinus* in citric acid.

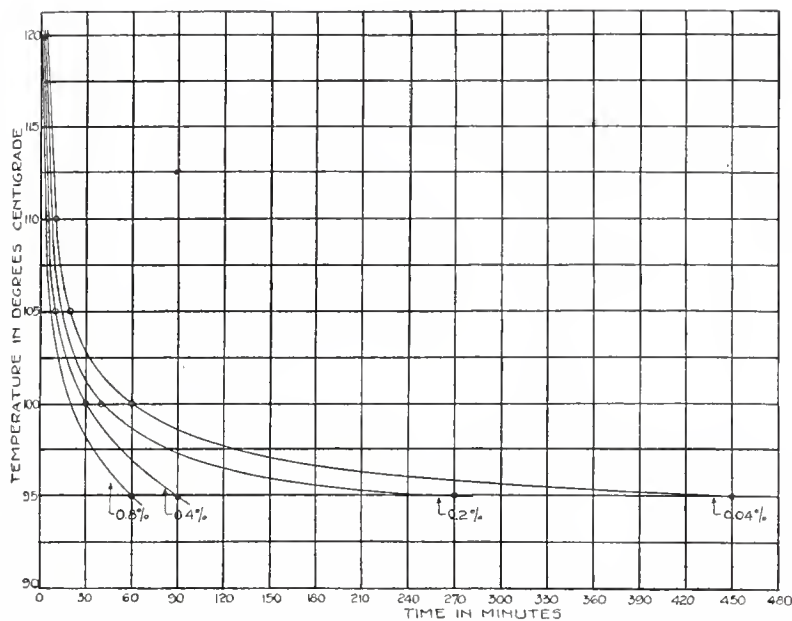


Chart 9.—Thermal death rate of spores of *B. botulinus* in sodium hydroxide.

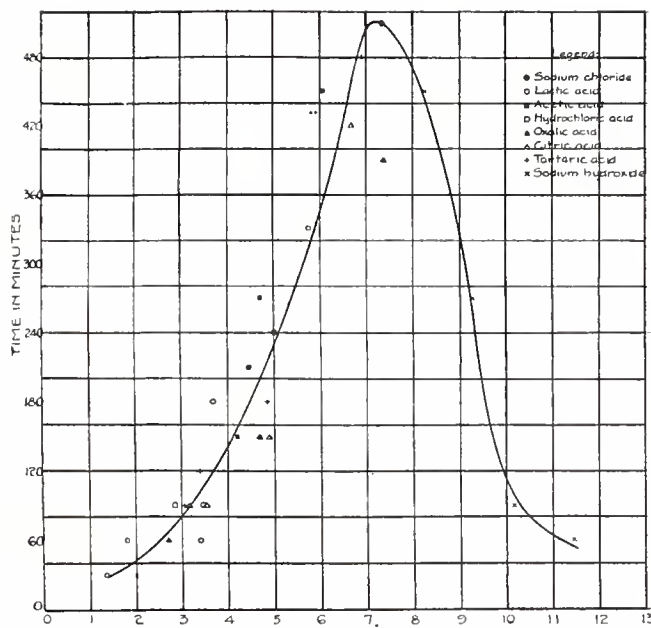


Chart 10.—Change in the thermal death point at 95 C. of spores of *B. botulinus* with change in PH value.

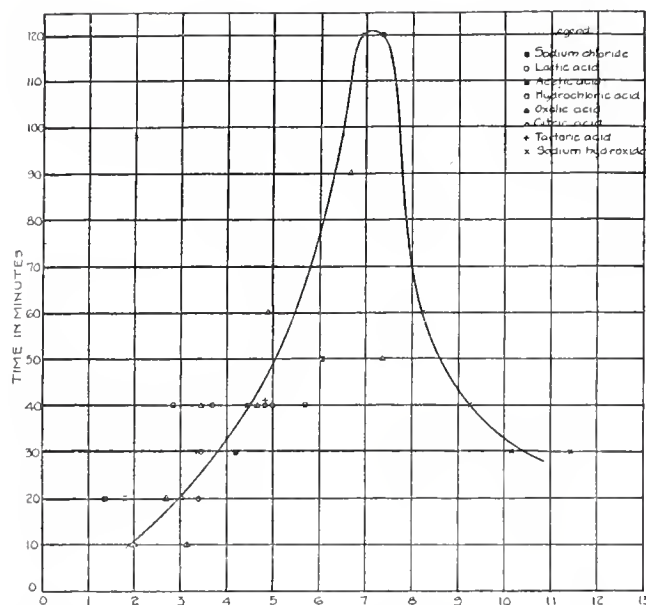


Chart 11.—Change in the thermal death point at 100 C. of spores of *B. botulinus* with change in P^H value.

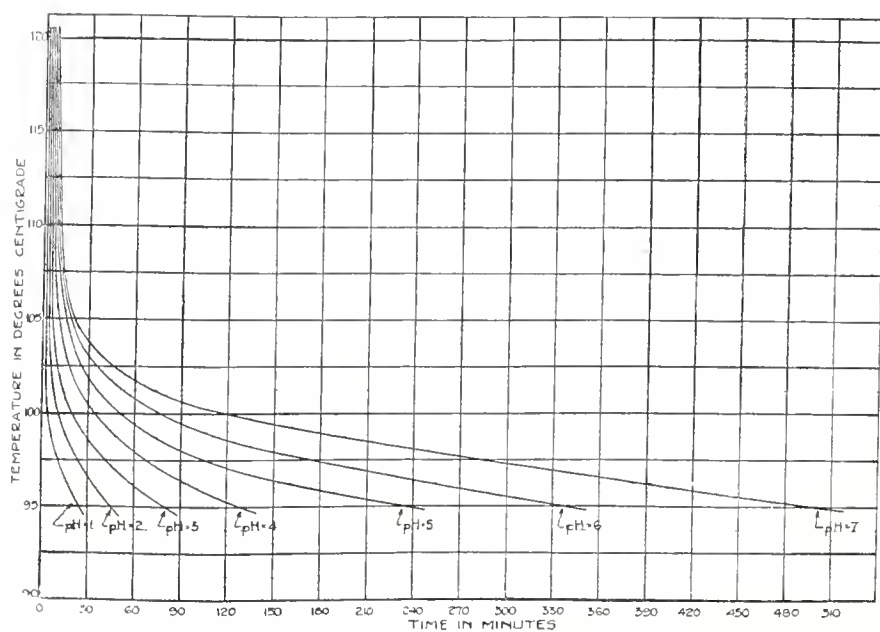


Chart 12.—Thermal death rate of spores of *B. botulinus* at P^H values 1 to 7, inclusive

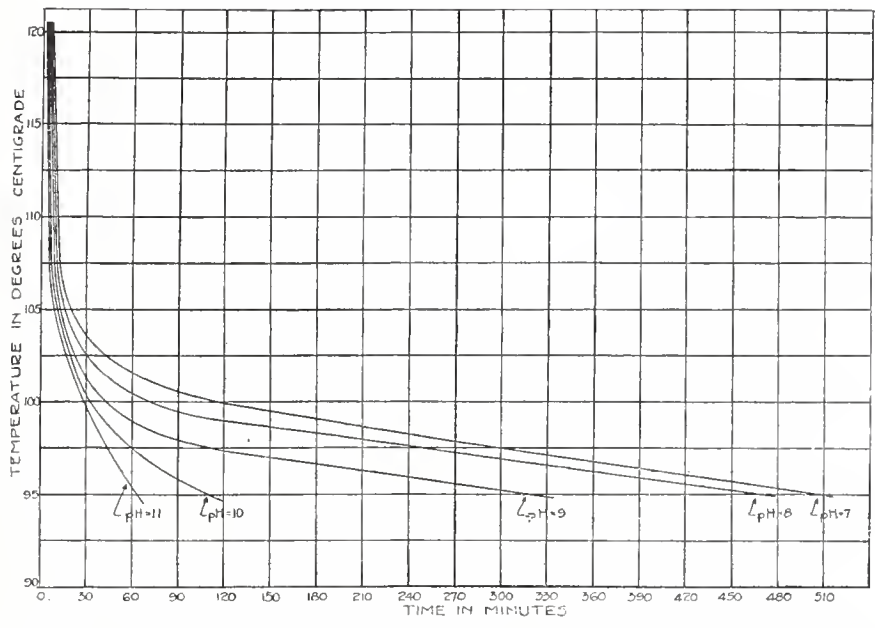


Chart 13.—Thermal death rate of spores of *B. botulinus* at P_H values 7 to 11, inclusive.

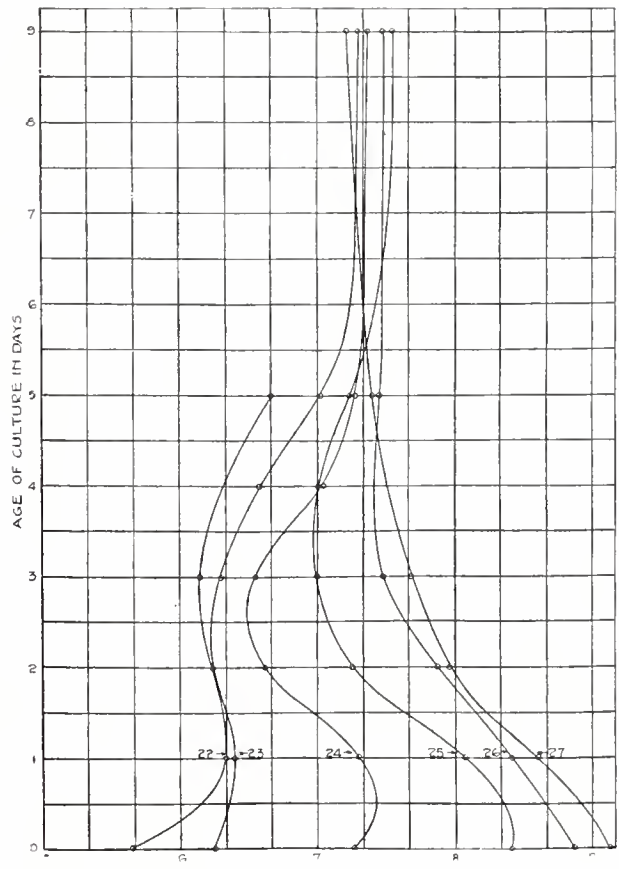


Chart 14.—Change in hydrogen-ion concentration during growth of *B. botulinus*.

TABLE 12
CHANGE IN HYDROGEN-ION CONCENTRATION IN SIX BOTTLES CONTAINING COAGULABLE MEAT PROTEIN AND 1% GLUCOSE

Medium No.	Uninoculated		1 Day		2 Days		3 Days		4 Days		5 Days		9 Days	
	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH	$\frac{+}{H}$	pH
22	2.3×10^{-6}	5.64	4.6×10^{-7}	6.34	6×10^{-7}	6.22	7.5×10^{-7}	6.13	2.2×10^{-7}	6.66	5×10^{-8}	7.30
23	6×10^{-7}	6.22	4×10^{-7}	6.40	5.8×10^{-7}	6.24	5×10^{-7}	6.30	2.7×10^{-7}	6.57	9.5×10^{-8}	7.03	4.5×10^{-8}	7.38
24	5.6×10^{-8}	7.25	5.3×10^{-8}	7.28	2.4×10^{-7}	6.62	2.8×10^{-7}	6.35	1.0×10^{-7}	7.00	5.8×10^{-8}	7.24	2.9×10^{-8}	7.54
25	4×10^{-9}	8.40	8.5×10^{-9}	8.07	6×10^{-8}	7.22	1.0×10^{-7}	7.00	9.5×10^{-8}	7.03	6×10^{-8}	7.22	3.3×10^{-8}	7.48
26	1.4×10^{-9}	8.85	4×10^{-9}	8.40	1.3×10^{-8}	7.89	3.3×10^{-8}	7.48	3.5×10^{-8}	7.46	3.3×10^{-8}	7.48
27	7.5×10^{-10}	9.13	2.5×10^{-9}	8.60	1.1×10^{-8}	7.96	2.1×10^{-8}	7.68	4.0×10^{-8}	7.40	6.5×10^{-8}	7.19

experiments at any temperature between 95 and 120 and at any P_H value in the acid range. Chart 13 gives the same for the alkaline range.

The P_H value must be determined immediately before the exposure is made. Should the spores of *B. botulinus* grow in a favorable medium the P_H value will change rapidly and ultimately reach the hydrogen-ion concentration at which it is most difficult to produce killing, that is, close to the neutral point.

In order to determine this effect, 6 bottles of meat infusion agar containing coagulable meat protein and 1% glucose were set at different hydrogen-ion concentrations and inoculated. The change in hydrogen-ion concentration was noted over a period of 9 days, when it became practically stable.

The changes are given in table 12 and are plotted in chart 14.

From these results it can be seen how essential it is to determine the P_H value just before sterilization on account of the rapidity of change and also since the general tendency is toward the value when a longer period of exposure is required.

Furthermore, for the practical purposes of canning, the heat penetration into the particular container used should be determined, and a sufficient factor of safety allowed.

SUMMARY AND CONCLUSIONS

The free spores of *B. botulinus* are destroyed within 5 hours at 100 C., within 40 minutes at 105 C., and within 6 minutes at 120 C. Bath temperatures are indicated. These thermal death points were determined under optimum conditions for survival.

The destruction of the spore is a gradual process, not an instantaneous killing and is probably due to a gradual protein coagulation. The spores are evidently injured before they are killed. This is inferred from the fact that the more protracted the period of heating, before killing occurs, the longer the period required for the spore to vegetate.

Young moist spores have a higher thermal resistance than old moist spores. Spores that are 1 month old are found to be three times as resistant as spores that are 5 months old.

There is a general decrease in thermal resistance as the spore ages. The more resistant individuals change more rapidly than the less resistant ones causing a tendency toward stabilization, the ultimate resistance

of the individual more nearly approximating the average resistance. This is shown by the effect of dilution on the thermal resistance of young and old spores, the old spore emulsions being practically unaffected by changes in the number of spores present, while the young spore emulsions show marked decrease in resistance as the dilution increases.

The thermal resistance of emulsions of young spores increases as the concentration of the emulsion increases.

Sodium chloride considerably lowers the thermal resistance and the rate of this lowering increases rapidly as the concentration of the salt is increased.

The hydrogen ion lowers the thermal resistance of the spore and the rate of this reduction decreases as the hydrogen-ion concentration increases.

The hydroxyl ion lowers the thermal resistance and the rate of the reduction decreases as the hydroxyl-ion concentration increases.

The hydrogen-ion concentration changes considerably in a medium in which *B. botulinus* is growing and ultimately stabilizes itself at a point near a P_H value of 7.5.

In applying these results to the practical problems of processing canned foods, it is necessary to determine the P_H value of the material to be sterilized immediately before the exposure. Any delay between the determination and the processing may cause a sufficient change in the P_H value to require a higher temperature or a longer period of exposure.

In all practical processing methods a sufficient safety factor should be allowed. The actual time required in applying such a factor becomes rapidly less as the temperature of processing is increased. Thus, a 50% safety factor applied at a processing temperature of 100 C., the medium to be sterilized having a P_H value of 7, would require an extra heating of 60 minutes or a total of 180 minutes. The same safety factor applied at a processing temperature of 120 C., the medium to be sterilized being the same, would require an extra heating of 3 minutes or a total of 9 minutes.

HEMOTOXIN PRODUCTION BY THE STREPTOCOCCUS IN RELATION TO ITS METABOLISM

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As hemotoxin¹ production by the streptococcus must be considered an evidence of metabolic processes, a study of this question involves a consideration of all factors, environmental and nutritional, which exert an influence on its growth. In fact, growth relationships regulate almost entirely the production of hemotoxin, for any change in the growth of the organism is reflected in the course of this production. By what process hemotoxin is formed is not known. Satisfactory evidence has been given that in the case of the streptococcus it is not an endotoxin, as bacterial extracts and preparations containing disintegrated organisms have not been found to possess hemotoxic properties. There has been considerable difference of opinion as to whether hemotoxin is produced as an entity, that is, whether it exists free from the organism. Experiments in which filtrates of hemotoxic cultures have been tested for their lytic properties have given variable results, as reported by different investigators. In a large number of cases, hemotoxic filtrates have not been obtained. However, according to Besredka,² M'Leod,³ and others, if the filtrate is prepared from a very active growth on a medium containing a large percentage of serum, from 20 to 50%, it possesses hemotoxic properties, although the concentration of hemotoxin of the filtrate is never as great as that of the original culture. These results establish the fact that hemotoxin under certain conditions may exist free from bacteria.

No conclusive evidence has been obtained in regard to the nature of hemotoxin, whether it is an enzyme, a secretion or excretion product. Extensive studies have been made of the chemical and

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¹ In view of our own experiments and in conformity with the usage of certain other authors (Sachs and Pribram), we prefer to refer to the blood corpuscle destroying substances in bacteria as hemotoxins rather than as hemolysins, since they differ markedly in their mode of action from the better defined hemolysin of normal and immune serums.

² Ann. de l'Inst. Pasteur, 1901, 15, p. 880.

³ J. Path. and Bacteriol., 1911-12, 16, p. 321.

physicochemical properties. It is known that hemotoxin is associated with the globulins of the medium, but as it has not been obtained in a pure state, its exact chemical nature has not been determined. As regards its physicochemical properties studies have been made of its thermolability, its dialyzing properties, the effect of acids, bases, and salts, as well as its behavior on inactivation and capabilities of reactivation. Concerning the immunologic properties of hemotoxin, there is also a difference of opinion, but the majority of workers have not been able to demonstrate antigenic properties, as satisfactory evidence of the formation of antihemotoxins has not been obtained.

A complete study of streptococcus hemotoxin would involve a consideration of all these phases of the subject. Of chief importance for the present work, however, is a study of the factors that govern the formation of hemotoxin in the living streptococcus culture. Among these factors are to be considered especially the influence of the composition of medium, the rate of growth, the relation between the presence of fermentable sugars and formation of hemotoxin and the effect of the metabolic products of the culture. Of the last, the question of the effect of acidity on hemotoxin production requires especial study. As virulence and growth relationships are closely connected, it is also of interest to determine whether passage of a culture through animals causes any variation in its hemotoxic properties. Experimental work on these phases of the subject will be reported.

EXPERIMENTAL PROCEDURE

The entire study was made on *Streptococcus pyogenes* "H," which was isolated from the lung in a fatal case of bronchopneumonia with empyema and endocarditis. This strain corresponds to culture 136 in a series of streptococci obtained in an investigation of pneumonia by Cole and MacCallum,⁴ and it has further served as the basis of an extensive study of experimental empyema in rabbits by Gay and Stone.⁵ For determinations on hemotoxin production two types of this culture were used—one that had been kept on artificial medium since the primary isolation and was called the laboratory strain, the other, a passage strain, that was injected intrapleurally into rabbits and recovered in the pleural exudate. Record of the type used is made in all experiments. In the present investigation no attempts were made to obtain hemotoxic filtrates; only living cultures were used.

⁴ Jour. Am. Med. Assn., 1918, 70, p. 1146.

⁵ J. Infect. Dis., 1920, 26, p. 265.

TECHNIC OF THE TITRATION OF HEMOTOXIN

All titrations of hemotoxin were made with sterile tubes, pipets, etc., to maintain the purity of the streptococcus culture. The corpuscles used were rabbit corpuscles, obtained by bleeding from the marginal ear vein into 0.85% sodium chloride containing 1% of sodium citrate. The corpuscles were washed 3 times in 0.85% sodium chloride and made up to a 1% suspension in beef infusion broth. To 0.5 c c of suspension were added amounts of culture varying from 0.005 to 0.5 c c. The volume was made up to 1 c c with broth and the mixtures incubated at 37 C. for 2 hours. During the first hour of incubation the tubes were shaken frequently to insure thorough mixing. The degree of lysis was observed at the end of the incubation period and the titer was expressed as follows:

100% hemolysis with 0.05 c c (or less) of culture.....	++++
100% hemolysis with 0.1 c c of culture.....	+++±
100% hemolysis with 0.25 c c of culture.....	+++
100% hemolysis with 0.5 c c of culture.....	++±
75% hemolysis with 0.5 c c of culture.....	++
50% hemolysis with 0.5 c c of culture.....	+±
25% hemolysis with 0.5 c c of culture.....	+
10% hemolysis with 0.5 c c of culture.....	±
0% hemolysis with 0.5 c c of culture.....	0

THE EFFECT OF THE MEDIUM ON HEMOTOXIN PRODUCTION

The question of the optimum composition of a medium for hemotoxin production has been studied by a large number of investigators. The conclusions made are uniform in that the maximum production has been found to occur in meat infusion broth containing a fairly high percentage of animal fluid, usually serum, although hydrocele and ascitic fluid have been found to be equally efficacious. It is generally conceded that a broth containing from 10 to 50% of horse serum furnishes the most favorable conditions for hemotoxin production (Lyll,⁶ M'Leod,³ Nakayama,⁷ Von Hellens⁸). Walbum⁹ lays particular emphasis on peptone. He considers that peptone is an essential factor and may be looked on as a prolysin. Lyll obtained better results with 2% than with 1% peptone. Sugars, particularly those that are easily fermented by the streptococcus, have been found by all workers to exert an inhibitory action on the production of hemotoxin.

⁶ J. Med. Res., 1914, 30, p. 315.

⁷ J. Infect. Dis., 1919, 25, p. 509.

⁸ Centralbl. f. Bakteriöl., O. I, 1913, 68, p. 602.

⁹ Ztschr. f. Immunitätsf., O., 1909, 3, p. 70.

In the present work beef infusion broth containing 1% of peptone and 0.5% NaCl was used as a basis for all mediums. No experiments were carried out to determine the effect of varying the concentration of peptone. Hemotoxin production was studied in plain beef infusion broth, in broths containing serum, carbohydrates and alcohols.

Exper. 1. Preliminary Test on Hemotoxin Production in Various Mediums.—Plain beef infusion broth, broth containing 5% rabbit serum,¹⁰ broth containing 1% glucose, and broth containing 5% serum and 1% glucose were inoculated with an 18-hour plain broth culture of the laboratory strain. The cultures were incubated for a period of 24 hours. Titrations of hemotoxin were made at the time of inoculation and at intervals of 1, 2, 4, 6, 8, 10, 12, 16 and 24 hours. The results are given in table 1, which shows that of the mediums tested, serum broth furnished the most favorable conditions for hemotoxin production. In the plain broth and serum glucose broth cultures, hemotoxin appeared later and in general was present in lower concentrations, while in glucose broth there was no hemotoxin demonstrable. In serum broth, hemotoxin was present in fairly high concentrations in a 1-hour growth, while the maximum production was reached at the fourth hour and persisted through the tenth hour. In plain broth, hemotoxin did not appear until the eighth hour, and it was then produced only in slight amounts. The maximum point of production in plain broth occurred at the tenth hour after which hemotoxin rapidly decreased. At no time did the concentration of hemotoxin in plain broth reach a point which was more than one-tenth as great as that in serum broth. In serum glucose broth the time of maximum production occurred at the tenth hour, as in plain broth. The concentration of hemotoxin was higher than in plain broth and lower than in serum broth.

TABLE 1
HEMOTOXIN PRODUCTION IN VARIOUS MEDIUMS

Age of Culture	Plain Broth	5% Serum Broth	1% Glucose Broth	5% Serum and 1% Glucose Broth
At inoculation	0	0	0	0
1 hour	0	+++	0	0
2 hours	0	+++	0	0
4 hours	0	++++	0	0
6 hours	0	++++	0	++
8 hours	+±	++++	0	+++
10 hours	++±	++++	0	++++
12 hours	++	++++±	0	++++±
16 hours	+±	++±	0	+++
24 hours	±	++±	0	+±

These results agree fairly well with those of other workers. Sekiguchi¹¹ found the first appearance of hemotoxin in a 1 to 2-hour growth of a serum broth culture. According to Lyall⁶ hemotoxin was not observed earlier than 3 hours, and, according to M'Leod,³

¹⁰ Rabbit serum was used in the early part of the work, but later was replaced by horse serum, comparative tests on the two showing no difference in their hemotoxin producing properties.

¹¹ J. Infect. Dis., 1917, 21, p. 475.

not earlier than 10 hours. The time of maximum production was found by Sekiguchi to be from 15 to 18 hours, by Von Hellens⁸ 14 hours, by Stevens and Koser¹² 6 to 10 hours and by de Kruif and Ireland¹³ 7 to 8 hours. All authors observed a gradual decrease beginning from 12 to 18 hours, with a final disappearance of hemotoxin in 48 to 72 hours. It is probable that the differences in the results of various investigators are due to the use of mediums of varying composition.

As in this preliminary experiment glucose was found to inhibit hemotoxin, a further test was made on the effect of this sugar and several other carbohydrates that are fermented by the streptococcus, of starch which is not digested by the "H" strain, and of the alcohols, glycerol and mannitol.

Exper. 2. Hemotoxin Production in Mediums Containing Various Carbohydrates and Alcohols.—A series of mediums containing glucose, levulose, lactose, starch, salicin, glycerol, and mannitol in concentration of 1%, as well as control preparations of plain broth and 5% serum broth, were inoculated in amounts of 10 cc with 0.1 cc of an 18-hour plain broth culture of pleural fluid 204. The cultures were incubated 24 hours, and portions removed at the beginning of incubation and at intervals of 4, 8, 12, and 24 hours, for tests on hemotoxin production. P_H determinations were also made to correlate the conditions of hemotoxin production with acidity production. The results are given in table 2.

It will be noticed in table 2 that as in exper. 1, the greatest production of hemotoxin occurred in a serum broth culture. In this experiment, the mediums next in order of efficiency were plain broth and glycerol broth. A retardation and decrease of hemotoxin production, as compared with serum and plain broth cultures, were observed in all mediums containing carbohydrates which are fermented by the streptococcus. The most marked inhibition occurred in cultures containing glucose and levulose. In lactose, starch, and mannitol mediums, hemotoxin production was almost equal to that in plain broth, while in salicin, a moderate amount of hemotoxin was formed. It will be noted that in this experiment the inhibition exerted by glucose was less marked than in exper. 1. This apparent discrepancy is probably due to the fact that the laboratory strain of streptococcus was used in exper. 1, while in exper. 2 the passage strain was used. Later experiments show distinct differences in results obtained with the two strains. Observation of the hydrogen-ion concentration of the

¹² J. Exper. Med., 1919, 30, p. 539.

¹³ J. Infect. Dis., 1920, 26, p. 285.

TABLE 2
HEMOTOXIN PRODUCTION IN MEDIA CONTAINING VARIOUS CARBOHYDRATES AND ALCOHOLS

Time	Plain Broth		Serum Broth		Plain Broth Containing													
					Glucose		Levulose		Lactose		Starch		Salicin		Glycerol		Mannitol	
	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu	Hem.	Pu
At inoculation	0	7.0	0	7.3	0	7.2	0	7.2	0	7.2	0	7.6	0	7.3	0	7.3	0	7.3
4 hours	+++	6.9	++++	7.1	+	7.1	++	7.1	+++	7.1	+++	7.5	+++	7.2	++	7.2	++	7.2
8 hours	++++±	6.9	++++	7.1	+++	5.95	+++	5.8	++++	6.1	+++	7.5	+++	7.0	++++	7.1	++++	7.2
12 hours	+++++	6.8	++++	7.1	++	5.05	++	5.0	++	5.9	++++	7.25	++	6.2	++++	7.1	+	7.2
24 hours	+++	6.6	++++	6.6	0	4.8	0	4.8	0	5.8	+++	7.2	0	5.9	++++	6.7	0	6.6

various mediums at the intervals recorded shows that hemotoxin was produced in smallest amounts when the acidity of the culture increased most rapidly.

Repetition of these tests did not give entirely constant results. Variations have been observed by a number of investigators. Lyall⁶ states that the interference by sugars is not always uniform, but that glucose is most constant in its inhibiting properties. Stevens and Koser⁹ found that glucose, maltose, and saccharose constantly inhibited, that lactose and salicin were inconstant. In the present work it was found that while there were differences in the extent to which glucose inhibited hemotoxin production, nevertheless a certain amount of inhibition was always exerted by this sugar, if used in a concentration of 1%. However, if lower percentages were used practically no inhibition was present, as will be seen in exper 3.

Exper 3. The Effect of Varying the Percentage of Glucose in the Medium Used for the Production of Hemotoxin.—Titrations of hemotoxin production were made on 6-hour cultures of streptococcus grown in plain broth containing 1, 0.1, and 0.01% of glucose, and in serum broth containing the same percentages of glucose. Control titrations on cultures in plain and serum broths containing no glucose were also made. For inoculation of these cultures 0.1 cc of a plain broth culture from pleural fluid 590 was added to 10 cc of each of the above mediums. The results were:

Percentage of Glucose	Plain Glucose Broth	Serum Glucose Broth
1.0	+	++++
0.1	++	+++++
0.01	+++	+++++
0.0	+++	+++++

It is evident that inhibition by glucose is not an absolute factor. It varies approximately with the concentration and is counteracted by the presence of serum. These results indicate that hemotoxin production or the lack of hemotoxin production in mediums containing glucose probably depends on the interaction of a number of influences in the growing culture, varying conditions of equilibrium among the several components producing varying results.

The mechanism of the inhibition of hemotoxin production by sugars is a question which has been the subject of much investigation. Kuhn¹⁴ has suggested that inhibition may be due to three factors: 1. A protein sparing action; 2. the prevention of the formation of a hemolytic ferment; 3. an injury to the activity of a hemolytic ferment. Of

¹⁴ Centralbl. f. Bakteriöl., O., I, 1912, 63, p. 97.

these, the two last would depend on the effect of acids formed. Stevens and Koser ¹² conclude that numerous interacting factors "affect streptolysin in the presence of fermented sugars. The principal action is the change in the metabolism of the streptococcus by which more carbohydrate and less protein is utilized; although the growth is much increased, there is proportionately less hemolysin. The acid developed in these cultures not only lessens the vitality of the growing organism and so lessens proteolysis, but is destructive to hemolysin at incubator temperature."

In considering the mechanism of inhibition by sugars, all further work has been confined to the action of glucose, as this sugar possesses the most marked and constant inhibiting properties. Study of the question follows the lines suggested by other workers, namely, the effect of acidity production and the accumulation of metabolic products, the relationship between rate of growth and hemotoxin production, the equilibrium between carbohydrate and protein metabolism in cultures which produce hemotoxin as compared with that of cultures in which hemotoxin production is inhibited. In connection with the last factor, the question of virulence has been found to be of some importance to hemotoxin production.

Before beginning an intensive study of these phases of the subject, it seemed advisable to determine whether the action of glucose was due to an inhibition of hemotoxin production in the living culture or whether the inhibitory influence was also operative in interfering with the lytic action of hemotoxin already formed.

Exper. 4. The Effect of Adding Glucose in Varying Concentration to Hemotoxin Titration Tubes.—To a number of tubes each containing 0.25 cc of a 12-hour culture of streptococcus grown in 5% serum broth varying amounts of glucose were added; 0.5 cc of a suspension of corpuscles and broth to make a total of 1 cc were added to each tube. The results after incubation were:

Glucose	Hemolysis
0.005 per cent	++++
0.01 per cent	++++
0.05 per cent	++++ change to purple color
0.1 per cent	++++ change to purple color
0.5 per cent	++++ change to purple color
1.0 per cent	++++ change to purple color
2.5 per cent	++++ change to purple color
Control 0	++++

A further control on the possible interfering action of glucose in the actual hemolytic test was made by exposing red cells to various concentrations of glucose before using them in the hemolytic test.

Exper. 5. The Effect of Suspending Corpuscles in Glucose Previous to Their Use in the Hemolytic Test.—Corpuscles which had been washed 3 times in 0.85% NaCl were treated as follows: Portion 1 was made up to a 1% concentration in NaCl containing 1% glucose, and portion 2 was made up to a 1% concentration in NaCl containing 10% glucose. Both portions were allowed to stand on ice for 16 hours, were then centrifugalized, and the corpuscles of each portion divided into 2 parts. The first part of each was washed 3 times in NaCl to remove all traces of glucose; the second part was not given further treatment. All four portions were made up to a 1% concentration in broth. The resulting suspensions, as well as a suspension of corpuscles prepared in the usual manner and used as a control, were added to a 5-hour culture of pleural fluid 4 in serum broth. The results were identical, and it is evident that glucose does not interfere with the lytic action of a hemotoxin once formed. The inhibition by glucose in cultures of streptococcus must, therefore, be due to its influence on the formation of hemotoxin.

THE EFFECT OF THE PRODUCTS OF GROWTH OF A GLUCOSE CULTURE ON HEMOTOXIN PRODUCTION

Having narrowed down the possibilities of inhibition of the hemotoxic activities of the streptococcus by glucose to its effect on the production of hemotoxin, consideration must be given to the various possible factors, previously mentioned, which may influence the formation of hemotoxin. Attention will first be given to the question of the influence on hemotoxin of the metabolic products of a glucose culture.

The time and rate of decrease of hemotoxin production in a glucose culture suggest that the accumulation of metabolic products may have a direct bearing on the inhibition of hemotoxin production, for hemotoxin in glucose cultures begins to diminish at approximately the time at which the lowering of the P_H of the culture indicates the presence of significant amounts of metabolic products, and ceases entirely before the maximum acidity is reached. To determine whether the inhibition of hemotoxin production may be ascribed to the presence of metabolic products by discovering whether removal of the metabolic products is followed by a resumption of hemotoxin production, organisms that were grown in glucose mediums were separated from the medium by centrifugation at a time when inhibition of hemotoxin production was complete. These organisms were then washed free from any traces of medium and resuspended in fresh mediums. Similar procedures were carried out with plain and serum broth cultures to control the possibility that the manipulations of the experiment had decreased hemotoxic activities.

Exper. 6. The Effect of the Removal of Metabolic Products on Hemotoxin Production.—Ten cc amounts of 12-hour cultures in plain, serum, and glucose broth, prepared as usual from pleural fluid 590, were centrifugated, and the supernatant fluids were removed. The sedimented organisms were washed 3 times in plain broth and resuspended in 2 cc of plain broth. This constituted a concentration of the culture 5 times. To 0.1 cc portions of each of these suspensions were added 0.4 cc amounts of plain, serum, and glucose broth, thus giving resultant suspensions that contained the same numbers of organisms as the original cultures. Hemolysis tests were made on these suspensions and on the supernatant fluids. Control tests were also made on portions of the cultures which were not centrifugated and on uninoculated portions of the mediums. The results of these tests are given in table 3.

TABLE 3
THE EFFECT OF RESUSPENDING CENTRIFUGATED CULTURES IN VARIOUS MEDIUMS

Material Tested for Hemotoxin	Amount	Medium Added	Hemolysis
Controls:			
Original glucose broth culture.....	0.5 cc	0	0
Original plain broth culture.....	0.5 cc	0	++++
Original serum broth culture.....	0.5 cc	0	++++
Uninoculated glucose broth.....	0.5 cc	0	0
Uninoculated plain broth.....	0.5 cc	0	0
Uninoculated serum broth.....	0.5 cc	0	0
Supernatant from glucose broth.....	0.5 cc	0	0
Supernatant from plain broth.....	0.5 cc	0	0
Supernatant from serum broth.....	0.5 cc	0	0
Sediment from glucose broth.....	0.1 cc	0.4 cc plain broth	++
Sediment from plain broth.....	0.1 cc	0.4 cc plain broth	+++
Sediment from serum broth.....	0.1 cc	0.4 cc plain broth	+++
Sediment from glucose broth.....	0.1 cc	0.4 cc serum broth	+++
Sediment from plain broth.....	0.1 cc	0.4 cc serum broth	+++
Sediment from serum broth.....	0.1 cc	0.4 cc serum broth	++++
Sediment from glucose broth.....	0.1 cc	0.4 cc glucose broth	0
Sediment from plain broth.....	0.1 cc	0.4 cc glucose broth	++
Sediment from serum broth.....	0.1 cc	0.4 cc glucose broth	+++

It appears that a glucose culture which is incapable of hemotoxin production at the period of 12 hours' growth, recovers hemotoxic properties to some extent when the organisms are removed from the surrounding medium and resuspended in medium which has not inhibiting properties on hemolysis, that is, in plain and serum broth. The increase in hemotoxic properties is most marked in the serum broth suspension. No lysis occurred in the portion of glucose culture resuspended in glucose broth. Tests on the hemotoxic properties of plain and serum broth cultures, which were subjected to the same treatment as the glucose broth culture, showed in the case of the plain broth culture that the sediments resuspended in plain broth and in serum broth were slightly less hemotoxic than the original culture, while the sediment from the serum broth culture was as active as the original cul-

ture when resuspended in serum broth but less active in plain broth. This would indicate that the manipulation of the culture involved in centrifugation lessened the hemotoxic activity of the organisms to some extent. Sediments from plain and serum broth cultures showed considerable activity when suspended in glucose broth. These results correspond fairly closely with those of exper. 4 in which the addition of glucose to cultures in which hemotoxin was already active caused no diminution of the hemotoxic properties. It is possible that the slight inhibition in the glucose suspensions of organisms grown in plain and serum broth in this experiment may be due to the lessening of hemotoxic activity due to centrifugation, which is manifest in all suspensions. No hemotoxin was present in any of the supernatant fluids. A repetition of this experiment gave the same results with the exception that the serum broth culture could not be completely sedimented by centrifugation, and the resultant supernatant fluid, as would be expected, was hemotoxic.

A number of points in regard to hemotoxin production by the streptococcus are brought out by this experiment, namely, that hemotoxin is not present free in the mediums used, as the supernatant fluids were not hemotoxic; and that hemotoxin is injured to some extent by centrifugation. This last detail is confirmed by the work of Robinson and Meader.¹⁵ The most significant fact is, however, that hemotoxic properties can be restored to a culture from which all hemotoxic properties have disappeared by removing the metabolic products. One must assume from this that the inhibition of lysis in glucose culture is due in a certain degree to degradation products in the medium. As a much longer time is required in plain and serum broth cultures for inhibition of hemolysis to occur, and as the P_H of these cultures decreases much less rapidly than in glucose, it is natural to assume that the acids formed in a glucose medium may be among the chief factors responsible for inhibition.

THE IMPORTANCE OF ACIDITY AS A FACTOR IN CAUSING INHIBITION OF HEMOTOXIN PRODUCTION IN GLUCOSE CULTURES

This question has been made the subject of investigation by many workers, as the possibility that a cause and effect relationship may exist between an increase in acidity and decrease in hemotoxin is one which readily suggests itself. In considering this subject a dis-

¹⁵ Proc. of Soc. of Amer. Bacteriol., 1919. Abst. Bacteriol., 1920, 4, p. 17.

tinction should be made between the effects of the initial acidity of the medium and of the acidity produced by the culture growing in glucose. The latter phase of the subject is the one suggested by the results obtained in exper. 6, but before entering into this question in detail mention should be made of the importance of the initial reaction to hemotoxin production.

According to the observations of Lyall,⁶ Stevens and Koser,¹² Robinson and Meader,¹⁵ hemotoxin may be produced in mediums having a fairly wide range of initial acidities. Lyall reports that hemotoxin production does not vary greatly in mediums of reactions varying from $+2$ to -2 . Beyond these limits no hemotoxin is produced. Robinson and Meader place the limits of hemotoxin production between P_H 7.2 and 5.3. Stevens and Koser found that in mediums, the P_H of which was decreased before inoculation by the addition of lactic acid, the growth of the streptococcus and the hemolysis produced were proportional to the P_H . All workers report scanty or no growth in cultures beyond the range of acidity or alkalinity at which hemotoxin is formed and consider that the initial acidity of a medium affects hemotoxin production only in so far as it affects growth.

An experiment carried out with strain "H" confirmed the results of others.

Exper. 7. The Effect of the Initial Acidity on Hemotoxin Production.—A series of tubes of plain broth varying in P_H from 8.35 to 5.05 was inoculated with an 18-hour plain broth culture of pleural fluid 1088. After 6 hours, growth was observed macroscopically, and titrations of hemotoxin production were made. The results follow:

Initial P_H	Growth	Hemolysis
5.05	0	0
5.35	0	0
5.85	0	0
6.15	+	\pm
6.4	++	++
6.9	++++	++++
7.3	++++	++++
7.55	++++	++++
7.9	++++	++++
8.1	++++	++++
8.35	++++	++++

It is evident that the initiation of the formation of hemotoxin may be entirely prevented by a high hydrogen-ion concentration, but the results also indicate, as has been suggested by others, that the inhibition is due to inhibition of growth and that hemotoxin production

is probably only indirectly affected. Hemotoxin is actively formed by cultures of P_H ranging from 6.9 to 8.35.

The second phase of the subject has to do with the action of the acids produced by growing culture. That hemotoxin usually disappears as acidity increases is an established fact, but whether the loss of hemotoxin is caused by the accumulation of acids or the two phenomena are merely coincident is not definitely known. There is considerable difference of opinion on this subject. Sekiguchi¹¹ assumes that hemotoxin formation is not hindered by the produced acids, since in his work "the acid and hemolysis curves do not bear the proper relation to each other." Sachs¹⁶ observed a simultaneous increase in acidity and decrease in hemotoxin, but questioned any direct effect of acidity since a daily neutralization of acid formed in his cultures did not increase hemolysis. This argument would hardly appear to be valid in view of the large amounts of acid which are formed in a day and the ample time before neutralization for these acids to act on the hemotoxin. Lyall⁶ calls attention to the fact that mere increase in acidity cannot be considered to be solely responsible for inhibition of hemotoxin since the inhibition in various sugar mediums is not absolutely proportional to the amounts of acid formed. Tubes showing the same amount of acid gave at the same time different concentrations of hemotoxin. This fact, together with the observation that in his experiments glucose alone exerted a constant inhibition, caused Lyall to advance the opinion that it is the kind rather than the amount of acid which is important. He showed that the acids formed as a result of the growth of the streptococcus have a different effect than HCl. Stevens and Koser¹² think that the acidity of a culture acts through growth inhibition, thus preventing the formation of hemotoxin, and also through a destruction of the hemotoxin already formed. Using hemolytic filtrates they showed that the effect of acid on the hemolysin was to destroy it, as sterile hemolytic filtrates incubated in various strengths of acid lost a large part of their hemolytic properties.

In the present work, attempts to show whether a causal relationship exists between acidity production and the inhibition of hemotoxic properties of a culture have taken the following lines:

1. Neutralization of acidity in the growing culture, (a) by the addition of phosphates, (b) by the addition of alkali.

¹⁶ Ztschr. f. Hyg. u. Infektionskr., 1909, 63, p. 463.

2. Neutralization of the acidity of a filtrate of a culture from which hemotoxin has disappeared, and reinoculation of the neutralized filtrate with a fresh culture.

Exper. 8. The Effect on Hemotoxin Production of the Addition of Dipotassium Phosphate to Glucose Cultures.—To beef infusion broth containing 1% glucose was added, in one series, 1% of K_2HPO_4 and in a second series 2% of K_2HPO_4 . Plain broth was also made up to the same concentration with phosphate, to control the effect of the phosphate itself on hemotoxin. Cultures from pleural fluid 4 were planted in these mediums in the proportion of 0.2 c c of culture to 10 c c of medium. Cultures were also made in glucose and plain broth containing no phosphate. All mediums were adjusted to approximately the same P_H before inoculation. Lysis titrations and P_H determinations were made on all cultures and on an uninoculated portion of each medium immediately after inoculation and at intervals of 6 and 12 hours. The results are given in table 4.

TABLE 4
RESULTS OF LYSIS TITRATIONS AND P_H DETERMINATIONS

Culture	Immediately After Inoculation		After 6 Hours' Incubation		After 12 Hours' Incubation	
	P_H	Hemolysis	P_H	Hemolysis	P_H	Hemolysis
Glucose broth containing 1% phosphate..	7.4	0	7.2	+++±	6.4	++++
Glucose broth containing 2% phosphate..	7.4	0	7.3	+	7.2	+++
Glucose broth (no phosphate).....	7.4	0	6.4	+++	5.2	0
Plain broth containing 1% phosphate.....	7.4	0	7.3	++++	7.25	++++
Plain broth containing 2% phosphate.....	7.4	0	7.3	++++	7.3	++++
Plain broth (no phosphate).....	7.4	0	6.8	++++	6.6	++++

Titration of the uninoculated medium gave no change in P_H and no evidence of lysis at any time.

A repetition of this experiment gave corresponding results. Evidently, the maintenance of an approximately neutral reaction in a glucose culture causes hemotoxin to persist for a longer time than in a culture in which the P_H decreases. Glucose culture containing 1% and 2% of phosphate gave strong hemolysis with a 12-hour growth, whereas glucose cultures without phosphate showed no hemolysis at this time. The concentration of hemotoxin in the 2% phosphate glucose culture is difficult to explain as the growth was good in this tube at both times the test was performed. Moreover, a 2% concentration of phosphate in plain broth gave the same titer of hemotoxin as tubes containing 1% and no phosphate. Phosphate itself, therefore, cannot be considered to have any direct effect on hemolysis. The prevention of an appreciable increase in acidity must be responsible for prolonging the action of hemotoxin. These results would indicate

that acidity must be considered as a factor concerned in the inhibition of hemotoxin by glucose.

In this experiment the action of phosphate was continuous during the growth of the culture, and it is probable that hemotoxin was almost entirely protected from the action of acid. In the next experiment, it was proposed to neutralize the acids formed in a glucose culture at frequent intervals, making the reaction correspond to that of a serum culture of the same age in which active hemotoxins were present. In connection with this, at the same intervals, acid was added to a serum culture to approximate as closely as possible the conditions in the glucose culture. The effect on hemolysis in both cultures was observed. Theoretically, it would seem possible that a glucose culture kept at the P_H of a serum culture might also follow to a certain extent the hemotoxin production of a serum culture, and that the serum culture in which the P_H was made to correspond to that of a glucose culture might show a corresponding loss in hemotoxin titer.

Exper. 9. The Effect on Hemotoxin Production of Adjustment of the Reaction of Glucose and Serum Cultures.—Two flasks of 1% glucose broth and 2 flasks of 5% serum broth, each containing 25 c.c. of medium, were inoculated with 1.5 c.c. each of a 12-hour plain broth culture from pleural fluid 590. Hemotoxin titrations and P_H determinations were made after 4, 6, 8, 10, and 24 hours of growth. Flask 1 of glucose broth and flask 1 of serum broth were allowed to follow their natural course of acidity production. Flask 2 of glucose broth was adjusted in reaction at the hours mentioned to the P_H of flask 1 of serum broth, while flask 2 of serum broth was adjusted to the P_H of flask 1 of glucose broth. Adjustments were made by the addition of HCl and NaOH. The results are recorded in table 5.

TABLE 5
RESULTS OF EXPER. 9

Period of Growth	Glucose Broth Culture					Serum Broth Culture				
	Flask 1		Flask 2			Flask 1		Flask 2		
	Hemotoxin	P_H	Hemotoxin	P_H	P_H Adjusted to	Hemotoxin	P_H	Hemotoxin	P_H	P_H Adjusted to
Before inoculation	0	6.8	0	6.8		0	6.8	0	6.8	
4 hours	++	5.5	++	5.5	6.8	++++	6.8	++++	6.8	5.5
6 hours	++	5.2	++	5.2	6.8	++++	6.8	++++	5.5	No change made
8 hours	++	5.2	++	5.2	6.8	++++	6.8	++++	5.5	No change made
10 hours	0	5.2	+	5.2	7.0	++++	6.8	++++	5.4	No change made
24 hours	0	5.0	0	5.2		++++	6.5	0	5.4	No change made

The results of this experiment are not striking. No differences occur between flasks 1 and 2 of the glucose cultures until the tenth

hour and not until after the tenth hour in the serum culture. The persistence of hemotoxin in the glucose culture, adjusted to the reaction of the serum culture, was only slightly greater than in the unadjusted culture. At no time did the hemotoxin production in the adjusted glucose culture equal that of the serum culture, and for the first 8 hours it did not exceed that of the unadjusted glucose.

In considering the lack of effect on hemotoxin production when the acidity of the glucose culture is neutralized by the addition of acid, note should be made of the rapid return to an acid reaction in the adjusted culture. It is probable that the effects of neutralization persisted for only a short time, as a subsequent experiment has shown that in a rapidly growing glucose culture a lowering of P_H of 0.6 occurs in half an hour after adjustment and that repeated adjustment is followed each time by the same rapid increase in acidity. The conditions in the flask of glucose culture in which the reaction was adjusted were not very different, then, from those of the unadjusted flask and a marked effect on hemotoxin could not therefore be expected.

Observation of the hemotoxin titer of the serum broth culture, which was adjusted to the P_H of the glucose broth culture, shows that hemotoxin is not destroyed, at least, and is perhaps still produced when exposed to a P_H of 5.5 during a period of 6 hours, that is, from the fourth to the tenth hour of growth. Comparisons should hardly be drawn, however, between the conditions in the flask of adjusted serum broth and the acidity produced by a growing culture, for the character of acid in the two is probably different, and as Lyall⁶ has suggested, the inhibiting action of the acids formed by the growth of bacteria in the presence of glucose may be qualitatively quite different from the effect exerted by HCl.¹⁷

With this point in mind a similar experiment was carried out using lactic acid to lower the P_H of the serum culture, as lactic acid is one of the degradation products of glucose.

Exper. 10. Further Observations on the Effect of Adjustment of Reaction on Hemotoxin Production.—The procedure of exper. 9 was followed, except that a glucose serum culture was substituted for a plain glucose culture and lactic acid was used in place of HCl. Pleural fluid 4 was the source of culture for inoculation (table 6).

The results of this experiment do not differ from those of the previous experiment. Repeated neutralization of the acidity of a

¹⁷ An investigation of the acids formed by glucose cultures of the streptococcus is now being made by L. F. Foster and G. Bernice Rhodes of this laboratory.

glucose culture caused a somewhat longer persistence of hemotoxin than occurred in a culture in which the reaction was not adjusted. The difference was slight, however. The results with serum broth culture were the same as in exper. 9. Lactic acid produced no effects different from those obtained with HCl. Both series of results indicate inhibition of hemotoxin production by acid.

TABLE 6
RESULTS OF EXPER. 10

Period of Growth	Glucose Serum Culture				Serum Culture				
	Flask 1		Flask 2		Flask 1		Flask 2		
	Hemo-toxin	P _H	Hemo-toxin	P _H Ad-justed to	Hemo-toxin	P _H	Hemo-toxin	P _H	P _H Ad-justed to
Before in-oculation	0	7.4	0		0	7.4	0	7.4	
3 hours	++	7.1	++	7.3	++++	7.35	++++	7.3	7.1
5 hours	++++	6.4	++++	7.0	++++	7.0	++++	6.8	6.4
7 hours	++++	5.7	++++	6.9	++++	6.9	++++	6.4	5.7
10 hours	++++	5.3	++++±	6.9	++++	6.9	++++±	5.6	5.3
12 hours	++	5.4	++++±	6.9	++++	6.8	++	5.3	No change made
24 hours	0	5.4	±		++++	6.85	0	5.4	

The effect of acidity on hemotoxin production was further studied by determining whether a neutralized filtrate from a glucose culture, which had ceased to produce hemotoxin, would again become hemotoxic if reinoculated with a fresh culture. If hemotoxin could be produced in such a neutralized filtrate, but not in a similarly inoculated but unneutralized filtrate, evidence would be furnished that the acids are the most effective metabolic products in inhibiting hemotoxin production.

Exper. 19. Further Observations on the Effect of Adjustment of Reaction Culture.—A flask containing 200 cc of a 1% glucose broth was inoculated with a 12-hour plain broth culture of pleural fluid 1088. Tests were made on this culture at intervals, and when hemotoxin was no longer demonstrable, the culture was filtered. A P_H determination was made on the filtrate, after which a 10 cc portion was reserved without neutralization, while the remainder was neutralized with sterile N/1 NaOH. Both portions were inoculated with 0.1 cc to every 10 cc of a 12-hour plain broth culture of pleural fluid 1088. After 6 to 8 hours' incubation tests were made as on the original culture. If lysis occurred, the filtrate culture was allowed to continue to grow until no hemotoxin was present. It was then filtered again. This procedure was repeated until a filtrate was obtained in which hemotoxin was not formed. In every case the P_H to which the filtrates were adjusted was 7.6. A qualitative determination of glucose by means of Benedict's test was made on the

original broth and on each successive filtrate before inoculation to give a rough estimation of the utilization of sugar (table 7).

TABLE 7
THE EFFECT OF NEUTRALIZATION ON HEMOTOXIN PRODUCTION IN FILTRATES OF
GLUCOSE CULTURES

Culture in	Growth	Test for Glucose*	Final Ph	Hemolysis	Period of Persistence of Hemotoxin
Original broth.....	++++	++++	5.2	++±	Between 10 and 12 hours
Filtrate 1					
Unneutralized.....	±	++++	...	±	Between 3 and 6 hours
Neutralized.....	++++	++++	5.05	++++	Between 6 and 19 hours
Filtrate 2					
Unneutralized.....	0	+++	...	0	
Neutralized.....	++++	+++	5.1	++++	Between 9 and 24 hours
Filtrate 3					
Unneutralized.....	0	±	...	0	
Neutralized.....	++++	±	6.85	++++	Between 24 and 48 hours
Filtrate 4					
Unneutralized.....	0	0	...	0	
Neutralized.....	++	0	7.4	+++	Between 6 and 24 hours
Filtrate 5					
Unneutralized.....	0	Not done	...	0	
Neutralized.....	+	Not done	7.4	+++	Between 6 and 24 hours

* The test for glucose was made in each case before inoculation.

These results show that while hemotoxin was not produced in unneutralized portions of the filtrate from a glucose culture, with the exception of a slight amount of hemotoxin in the first filtrate, the neutralized filtrate becomes an excellent medium for hemotoxin production when reinoculated with a fresh culture. Not only is the first filtrate capable of furnishing material for hemotoxin production, but the second and third filtrates are equally efficacious. In fact, as the glucose was progressively removed from each successive filtrate, hemotoxin production increased over that in the original broth up to the fourth filtrate. Growth of the organism was likewise rapid in the first 3 filtrates. In the fourth and fifth filtrates both growth and hemotoxin production decreased. From these data one may conclude that of the metabolic products the acids are most active in inhibiting hemotoxin production. It is probable that with the rapid utilization of the glucose in the medium, the degradation products from the sugar form the greater part of all the products of metabolism, since the proteins are undoubtedly spared at the expense of the glucose. As long as glucose is present in the medium, the metabolic products chiefly responsible for inhibition of hemotoxin are the acids formed. With the continued utilization of glucose up to a point of disappearance, other elements of the medium must be called on to supply the growth and energy

requirements of the organism. After this period the nature of the metabolic products must change. As no efforts were made to remove any of the cleavage substances, other than acids, a gradual accumulation of all other products of metabolism must have been made with each successive filtrate. From observation of table 7 it would appear that such an accumulation, slight during the cleavage of glucose, exerted an appreciable influence in the fourth and fifth filtrates, since growth became less active and the length and intensity of production of hemotoxin decreased considerably.

This experiment indicates that in a glucose culture, the metabolic products responsible for the inhibition of hemotoxin production are the acids. With the elimination of glucose, there is probably an increased utilization of the protein constituents of the medium with the formation of nitrogenous degradation products. Sufficient accumulation of these substances also exerts an inhibiting action on hemotoxin production. In the usual tests on hemotoxin production in a 1% culture, however, the action of the nitrogenous degradation products must be almost negligible, as hemotoxin production ceases before sufficient glucose has been removed from the medium to permit an increase in the protein metabolism.

To summarize the results of the three types of experiment, acidity must be considered a most important factor in the inhibition of hemotoxin, since (1) continuous neutralization of the acids formed through the use of phosphates prolongs the period of production of hemotoxin; (2) frequently repeated, but not continuous, neutralization exerts a slightly favorable effect on hemotoxin production, while the addition of hydrochloric and lactic acids to a serum culture decreases the period of hemotoxin production, and (3) neutralization of the acidity of a filtrate from a glucose culture restores to the medium its hemotoxin producing properties, while hemotoxin is not obtained from an unneutralized portion. However, although acidity has a great influence on the inhibition of hemotoxin production in glucose cultures, it is probably not the only contributing factor. The fact that in two tubes, having the same hydrogen-ion concentration, there may be different quantities of hemotoxin produced is evidence that other forces also exert an influence. The rate of growth is unquestionably of importance. During the course of all experiments in which glucose cultures were used, it was repeatedly noted that a rapidly growing culture with a P_H lower than that of a culture in which growth was not so luxuriant

had, nevertheless, a higher hemotoxin titer. A study was therefore made of the relationship of the rate of growth to hemotoxin production in glucose cultures.

THE EFFECT OF RATE OF GROWTH ON HEMOTOXIN PRODUCTION
IN GLUCOSE CULTURES

The view is uniformly held by investigators who have studied the question of hemotoxin production that in mediums of uniform and constant composition the concentration of hemotoxin is to a large extent dependent on the rapidity of multiplication of organisms. Besiedka² states that the potency of a hemotoxin depends on the abundance of growth. M'Leod³ found that hemolysin production increased with the rapidity of multiplication. Sachs,¹⁶ Pribram,¹⁸ Rieke,¹⁹ and Lyall⁶ express similar views—that hemotoxin production is a function of the increasing and multiplying organism and that maximum amounts are produced by young, active cultures. Closely connected with the rapidity of growth is the amount of inoculum used. This question is of particular importance to hemotoxin production in glucose cultures, for hemotoxin is formed in the early periods of growth only, and any factor that diminishes the lag of a culture will presumably have a direct influence on the formation of hemotoxin. Rieke¹⁹ and Lyall⁶ found considerable differences in the hemotoxin production of cultures in the same medium which were inoculated with different amounts. In cultures inoculated heavily, when growth was initiated with practically no lag, hemotoxin appeared earlier and in higher concentration than in cultures lightly inoculated. In our preliminary experiments certain variations in the production of hemotoxin in glucose cultures can probably be ascribed in part at least to differences in the amount of inoculum. This question is considered in detail in the following experiment.

Exper. 12. The Relation Between the Rate of Growth and Hemotoxin Production in a Glucose Medium.—Two tubes containing 1% glucose broth were inoculated, the first with 3 c c of a 16-hour plain broth culture from pleural fluid 4 and the second with 0.3 c c of the same culture. Both tubes after inoculation contained the same amount of material—20 c c. Hemolysis tests, counts by the plating method, and P_H determinations were made after 4, 7 and 10-hour periods of growth (table 8).

This experiment was repeated a number of times and in every case the same relationship was found to exist between growth and hemotoxin production.

¹⁸ Handbuch der Path. Mikroorganismen, 1913, 2, p. 1328.

¹⁹ Centralbl. f. Bakteriol., O., I, 1904, 36, p. 321.

A number of interesting points are brought out by a study of these results. In the heavily inoculated tube, in spite of a rapid lowering of P_H hemotoxin production proceeded for a time at a rate almost parallel with the rate of growth. At 4 hours there was a high titer of hemotoxin in this culture while in the lightly inoculated culture there was no hemotoxin. At the seventh hour the hemotoxin titer of the two cultures was particularly interesting.

TABLE 8
THE RELATION BETWEEN RATE OF GROWTH AND HEMOTOXIN PRODUCTION

Age of Culture	Tube Inoculated With 3.0 c c Culture			Tube Inoculated With 0.3 c c Culture		
	Hemotoxin	Count— Millions per C.mm.	P_H	Hemotoxin	Count— Millions per C.mm.	P_H
0 hours	0		7.6	0		7.6
4 hours	++++	1,162	6.0	0	0.9	6.8
7 hours	+++	1167.0	5.6	++	1.2	6.4
10 hours	0	80,000.0	5.2	+	710.0	5.6

In the heavily inoculated culture with a count of 1,167 million per c. mm., hemotoxin was present in the concentration of +++, although the P_H was 5.6. In the lightly inoculated culture when the P_H was 6.4, but when the number of organisms was approximately equal to those giving a ++++ hemotoxin titer at the fourth hour in the heavily inoculated culture, the titer was only ++. Moreover, this was the point of maximum production by this culture, since at the tenth hour the titer had decreased to + although the actual number of organisms was still increasing. From these data, it would appear that in a glucose culture the presence or lack of lag and the rapidity of multiplication regulate to some extent the production of hemotoxin. In a steadily but slowly increasing culture only slight amounts of hemotoxin are formed. Probably in all cultures we are concerned with the interaction of opposing forces. In the heavily inoculated culture the factors favoring hemotoxin production, namely, the rapid rate of multiplication of organisms, outweigh the inhibitory influence of glucose, while in the lightly inoculated culture the rate of growth is not sufficiently rapid at any time to overcome the forces acting simultaneously to inhibit. Even in the heavily inoculated culture, however, the rate of growth is not sufficient to maintain a high degree of hemotoxin production, for at the seventh hour when the organisms are still actively multiplying, the hemotoxin titer decreases and at the

tenth hour has entirely disappeared. One may conclude that the rate of growth has a direct influence on hemotoxin production, but as growth is only one of several factors operating it is not possible to show the exact relationship existing between the two.

THE EFFECT OF VIRULENCE ON HEMOTOXIN PRODUCTION

As the rate of growth of a culture of streptococcus in a glucose medium is of so much importance to the production of hemotoxin, the question of the relation of virulence to hemotoxin production was suggested. A comparison of the results obtained in expts. 1 and 2 shows that a culture of the Holden strain, which had not been passed through animals, differed in hemotoxic properties from the same strain after repeated passage through the pleura of rabbits. The first, or laboratory, strain never produced hemotoxin in a glucose broth culture, while the passage strain usually was hemotoxic during the early period of growth on glucose. As the animals used for maintaining the virulence of the culture were rabbits and as the corpuscles used in all hemolytic tests were rabbit corpuscles, it seemed possible that the difference between the laboratory culture and the passage culture might be due to a specific increase in virulence for rabbit corpuscles, which would not be effective with corpuscles of other animals. A test was accordingly made to show the comparative activities of the laboratory and passage cultures on various corpuscles.

Expt. 13. A Comparison of the Hemotoxic Action of Laboratory and Passage Cultures on Corpuscles of Different Sources.—From a serum-broth culture of the laboratory strain and from pleural fluid 204, subcultures were made in plain and serum broths. On 10-hour growths of these cultures, hemotoxin titrations were made, using in 4 series of tests, rabbit, sheep, guinea-pig, and human corpuscles. The results were:

	Rabbit	Sheep	Guinea-Pig	Human
Laboratory strain in plain broth.....	+++	+++	++	±
Laboratory strain in serum broth.....	++++	++++	++++	++
Pleural fluid 204 in plain broth.....	++++	++++	+++	+++
Pleural fluid 204 in serum broth.....	++++	++++	++++	+++

Evidently the passage strain is somewhat more hemotoxic for all kinds of corpuscles than the laboratory strain. Both strains act more readily on rabbit and sheep than on human and guinea-pig corpuscles. As the comparative differences are the same in both cultures, however, they can hardly be ascribed to a selective virulence for rabbit corpuscles.

The differences between the laboratory strain and the passage strain, as evidenced by the lack of hemotoxin production in glucose broth by the laboratory strain and the ability to produce hemotoxin in this medium by the passage strain, must depend, therefore, on factors other than a specific increase in activity for rabbit corpuscles. It is possible that these differences are due to changed properties of growth through cultivation on artificial medium on the one hand and actual experimental infection on the other. One would expect that such differences would be manifest in some phase of the metabolism of the two organisms, such as a variation in the utilization of protein, or in the fermentative properties. To determine whether this is the case, various phases of the metabolism of the two strains were investigated. As a means of orientation in a preliminary experiment in which mediums containing a number of carbohydrates and alcohols were used, the hemotoxic and fermentative activities of the two strains were compared. In a further experiment, using glucose medium only, quantitative determinations of protein and carbohydrate metabolism were made. These experiments follow.

Exper. 14. A Comparison of the Hemotoxic and Fermentative Activities of the Laboratory and Passage Strains in Mediums Containing Various Carbohydrates and Alcohols.—Mediums containing 1% glucose, levulose, lactose, salicin, glycerol, and mannitol, and also plain and serum broth, were inoculated with proportionate amounts of plain broth cultures of the laboratory strain and pleural fluid 204. P_H determinations and hemotoxin titrations were made at the time of inoculation and at 4, 8, 14, and 24 hours of growth (tables 9 and 10).

It is evident that the laboratory strain possesses much less hemotoxic activity than the passage strain. In the serum and mannitol cultures, only, was the degree of hemotoxin production equal to that of the passage strain. The inhibiting action of sugars on the laboratory strain was complete in every instance but one (14-hour growth in lactose), while hemotoxin was formed to some extent in all cultures of the passage strain. In general, acid formation was less rapid in the laboratory strain than in the passage strain. The lack of hemotoxin production with this strain in the presence of fermented sugars was not due, then, to a greater concentration of acids.

Exper. 15. A Comparative Study of the Metabolism of the Laboratory and Passage Strains.—Of 3 flasks, each containing 200 cc of 1% glucose broth, the first was inoculated with 10 cc of an 18-hour plain broth culture of the laboratory strain, the second with 10 cc of an 18-hour plain broth culture of

TABLE 9
HEMOTOXIC AND FERMENTATIVE ACTIVITIES OF THE LABORATORY STRAIN IN VARIOUS MEDIUMS

Time	Type of Medium															
	Plain		Serum		Glucose		Levulose		Lactose		Salicin		Glycerol		Mannitol	
	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H
At inoculation	0	7.3	0	7.3	0	7.2	0	7.2	0	7.2	0	7.3	0	7.3	0	7.3
4 hours	Purple	7.1	+++	7.1	0	7.1	0	7.1	0	7.1	0	7.3	0	7.3	0	7.3
8 hours	+++	6.85	+++	7.65	0	6.5	0	6.55	0	6.6	0	7.1	0	7.15	+	7.15
14 hours	+++	6.85	+++	7.0	0	5.1	0	5.6	++	6.6	0	6.0	0	7.15	+	7.15
24 hours	0	6.6	+++	6.5	0	5.0	0	5.6	0	6.6	0	5.8	0	6.6	0	6.6

TABLE 10
HEMOTOXIC AND FERMENTATIVE ACTIVITIES OF PLEURAL FLUID 204 IN VARIOUS MEDIUMS

Time	Type of Medium															
	Plain		Serum		Glucose		Levulose		Lactose		Salicin		Glycerol		Mannitol	
	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H	Hemo- toxin	P _H
At inoculation	0	7.0	0	7.3	0	7.2	0	7.2	0	7.2	0	7.3	0	7.3	0	7.3
4 hours	++	6.9	++	7.1	+	7.1	++	5.8	++	7.1	+	7.2	+	7.2	+	7.2
8 hours	+++	6.9	+++	7.1	++	5.95	++	5.8	++	6.1	0	7.0	+	7.1	+	7.1
14 hours	++++	6.8	++++	7.1	+++	5.05	+++	5.0	±	5.9	0	6.2	+++	7.1	0	7.1
24 hours	+	6.6	+	6.6	0	4.8	0	4.8	0	5.8	0	5.9	+++	6.7	0	6.7

pleural fluid 19, and to the third, which served as a control, 10 cc of sterile plain broth was added. The flasks were incubated. Fifty cc portions of medium were removed from each flask immediately after inoculation and at periods of 6 and 24 hours of growth, for determinations on the number of organisms, the P_H , the hemotoxin titer, glucose utilization and ammonia production. Wright's method of counting was used. The hemotoxin titrations and P_H determinations were made in the usual way. The utilization of glucose was measured by determining the quantity of glucose present in the medium at each period of growth, the loss from time to time representing the amount of sugar utilized. Bertrand's procedure for the determination of glucose was used. For this test a preliminary clarification of the culture to remove peptones was necessary, since peptones interfere with the reduction test. The broth was clarified as follows:

Twenty cc of culture was diluted 1:5 with distilled water to make a total of 100 cc. To this, 20 cc of a saturated aqueous solution of tannic acid was added. The precipitate which formed was removed by filtration. To the filtrate was added sufficient dry lead acetate to precipitate the tannic acid. This was again filtered and the excess lead acetate in the resulting filtrate was precipitated by the addition of dry sodium oxalate. On removal of this precipitate by filtration, a clear fluid was obtained which was peptone-free and was suitable for use in determining the amount of glucose in the culture under examination.

TABLE 11
TESTS ON THE METABOLISM OF THE LABORATORY AND PASSAGE STRAINS

Time	Hemo- toxin Titer	Count per C.mm.	P_H	Glucose		NH ₃ as	
				Gm. per 100 C c	Total Mg. Utilized	Mg. N per 100 C c	Total Mg. N Increase
Laboratory Strain							
0 hours	0	19,200	7.8	0.902	...	5.054	
6 hours	0	800,900	5.9	0.845	57	6.580	1.526
24 hours	0	1,125,500	5.35	0.772	130	6.510	1.456
Pleural Fluid 19							
0 hours	0	26,400	7.8	0.927	...	7.00	
6 hours	+++	1,159,660	5.3	0.804	123	7.196	0.196
24 hours	0	2,187,500	5.2	0.767	160	8.288	1.288
Uninoculated Con- trol							
0 hours	0	7.8	0.945	...	5.53	
6 hours	0	7.8				
24 hours	0	7.8	0.966	...	5.19	

Ammonia determinations were made according to the method of Kendall, Day, and Walker of the Northwestern University Medical School:

To 2 cc of broth culture were added 2 cc of a 5% solution of sodium oxalate containing sodium carbonate and 1 cc of hydrocarbon oil. Air washed in acid was forced through this mixture, slowly for the first 5 minutes, rapidly for the next 15 minutes. The ammonia liberated was collected in a measured amount of N/50 HCl (5 cc). Titration of the unneutralized acid was made with N/50 NaOH, using alizarin as indicator. Calculation of the NH₃ nitrogen was then made.

The results are given in table 11. A repetition of this experiment gave similar results.

The results of this experiment corroborate all the earlier observations on the differences in the ability of the two strains to produce hemotoxin in glucose medium. The passage strain gave a +++ titer of hemotoxin after 6 hours' growth, while no hemotoxin was produced at any time by the laboratory strain. This difference is paralleled to a certain extent by differences in other metabolic activities. During the first 6 hours—that is, during the period of hemotoxin production by the passage strain—this strain showed a greater rate of growth, a more marked acidity production, and a greater utilization of glucose than the laboratory strain. The difference in NH_3 nitrogen was not marked. Nevertheless, larger amounts were produced by the laboratory than by the passage strain during the first six hours. This would be expected in view of the greater utilization of glucose by the passage strain during this period. At 24 hours the 2 strains more nearly approached each other in all phases of their metabolism.

The points of especial interest brought out by this experiment are that hemotoxin was formed most actively by the passage strain during the period of most rapid utilization of glucose and the greatest production of acid. If the inhibition of hemotoxin production by glucose were due to a protein sparing action, as suggested by Kuhn¹⁴ and Stevens and Koser,¹² one would expect that hemotoxin would not be formed during this period. The results do not confirm this assumption. Evidently, it is not the utilization of glucose alone which interferes with hemotoxin production. A further contribution to the question of the relationship of acidity to hemotoxin production is also furnished by this experiment, since the passage strain gave a positive hemotoxic test at a time when the P_H was considerably lower than that of the laboratory strain.

Since the only explanation suggested by these results in regard to the differences in the hemotoxic activities of the laboratory and passage strains in the presence of glucose was the more rapid rate of metabolism of the passage strain in the early period of growth, it was considered of interest to determine whether the laboratory strain, which is known to produce hemotoxin in the presence of glucose if serum is also present in the medium, would approach the passage strain in other phases of metabolism when grown on glucose serum medium. Determinations were therefore made of the carbohydrate and protein metabolism of the laboratory and passage strains when grown on glucose serum broth.

Exper. 16. Further Comparative Studies of the Metabolism of the Laboratory and Passage Strains.—A procedure was employed similar to that of exper. 15. The medium used contained in addition to 1% of glucose, 5% of horse serum. The pleural fluid in this case was 198. A further measurement of metabolism was made by determining the amino acid content of the cultures by means of formol titrations. These titrations were performed according to the method employed by Kendall, Day and Walker. The procedure was as follows:

To 50 c c of distilled water were added 5 c c of a broth culture and 1 c c of phenolphthalein. This was titrated to neutrality with N/10 NaOH. Five c c of neutral formaldehyd were added, and the mixture titrated again. The amino acid content was determined from the last figure according to the equation $R.CH.NH_2 + HCOH \rightleftharpoons R.CH.N.CH_2 + H_2O$.

COOH

COOH

All determinations were made at the time of inoculation and at 3, 6, 9, and 12 hour periods of growth (table 12).

TABLE 12
METABOLISM OF LABORATORY AND PASSAGE STRAINS

Time	Hemo- toxin Titer	Count per C.mm.	Ph	Glucose		NH ₃ as Mg. N per 100 C c	Amino Acids as Mg. N per 100 C c
				Gm. per 100 C c	Total Mg. Utilized		
Laboratory Strain							
0 hours	0	40,000	7.35	0.992	...	6.43	19.17
3 hours	0	90,400	7.20	1.001	...	5.45	20.95
6 hours	+++++	1,456,000	5.90	0.862	130	8.67	18.03
9 hours	+++++	2,860,000	4.95	0.722	270	8.25	20.55
12 hours	0	4,700,000	4.90	0.728	262	7.69	20.61
Pleural Fluid 198							
0 hours	0	23,800	7.35	1.096	28	5.03	19.77
3 hours	0	90,200	7.20	1.068	28	6.29	18.21
6 hours	+++++	2,000,000	5.80	0.941	155	9.93	16.87
9 hours	+++++	3,500,000	5.10	0.877	219	10.49	19.41
12 hours	0	4,560,000	4.90	0.847	249	10.07	20.73

The results of this experiment show a much greater similarity between the 2 strains in serum glucose broth than in plain glucose broth. Both produced hemotoxin in the same concentration and at the same period of growth. The rates of growth and acidity production ran closely parallel. The passage culture used somewhat less glucose and produced somewhat greater amounts of NH₃ nitrogen than the laboratory strain, but the differences were not marked. The values obtained for amino acid nitrogen show a similarity between the 2 strains, in that both reached a low point at 6 hours, followed by a subsequent increase.

A consideration of the results of this experiment, together with those of exper. 15, brings out several important points. First, when the various phases of the metabolism of the laboratory and passage

strains are practically the same, there are no differences in hemotoxin production. Second, when the two strains are grown in plain glucose broth, a medium on which the rate of growth and other metabolic activities of the two strains differ, hemotoxin production occurs only in the strain which shows the greater rate of metabolism. When, by changing the conditions of growth of the laboratory strain, its metabolism is increased in every respect—that is, in rate of growth, acidity production, glucose utilization, and the production of NH_3 nitrogen, it becomes capable of producing hemotoxin in the presence of glucose. From these results it is evident that the ability to produce hemotoxin in a glucose medium cannot be based on any one phase of metabolism, since an increase in hemotoxic activity occurs simultaneously with an increase in all other types of metabolism. Certainly the hemotoxic action of the laboratory strain in exper. 16 cannot be due to the greater utilization of glucose and increased acidity production. It is probable, rather, that in the cultures in which hemotoxin was produced, a factor was operating which acted to form hemotoxin in spite of the increased carbohydrate metabolism. This factor can be described only as an increased vitality of the organism. Further evidence that hemotoxin production is closely related to the vitality of the organism is furnished by the fact that hemotoxin occurs in every case immediately following the period of greatest activity of the culture as measured by determinations of all types of metabolism.

The only explanation of the relationship between virulence and hemotoxin production given by these experiments is the general tendency for the passage strain to show greater activity than the laboratory strain in all phases of its metabolism. No one function is particularly emphasized. In regard to this question, Rosenthal and Patai²⁰ report similar results, except that in determinations of the metabolism of virulent and avirulent strains, these authors obtained a greater cleavage of amino acids by virulent than by avirulent strains. An insufficient number of formol titrations was made in the present work to draw any conclusions on this point.

CONCLUSIONS

This is a study on the hemotoxic and other metabolic properties of a single strain of human *Streptococcus pyogenes* (Holman) grown under two conditions: (1) artificial medium since isolation (laboratory

²⁰ *Centralbl. f. Bakteriol., O., I*, 1914, 73, p. 406.

strain); (2) the same passed repeatedly through the pleura of rabbits (passage strain). The following conclusions may be drawn:

There is a distinct difference in the hemotoxic properties of the laboratory and passage strains.

This difference is not due to differences in the rate of multiplication alone.

The inhibition of hemotoxin production in the laboratory strain as contrasted with the hemotoxic activity of the passage strain cannot be ascribed to a greater acidity production by the laboratory strain.

The inhibition of hemotoxin production in the laboratory strain cannot be ascribed to a greater protein sparing action as evidenced by a greater utilization of glucose.

The types of protein metabolism of the two strains are not sufficiently dissimilar to serve as an index to differences in hemotoxin production.

The production of hemotoxin by the laboratory strain in a glucose serum culture, as contrasted by lack of hemotoxin production in a glucose culture, cannot be ascribed to any one difference in metabolism, which was measured by the methods used above.

The only factor that can be said definitely to favor hemotoxin production is increased vitality of the organism.

The question of hemotoxin production remains a complicated one. A summary of the entire study indicates only that hemotoxin production is due to, and controlled by, conditions that influence growth. As such conditions can be studied only as a complex of interacting forces with no possibility of observing the action of a single variant, it is not possible to state the exact relationship between any one phase of metabolism and hemotoxin production. One can conclude only that hemotoxin production is an expression of the summation of activities of the organism and is capable of varying with every variation of environment and nutrition.

HEMOLYTIC STREPTOCOCCI OF THE APPENDIX VERMIFORMIS

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The significance of hemolytic streptococci in the etiology of appendicitis is not well known. Osler¹ says that the *Streptococcus pyogenes* is present in a large number of cases. Rose and Carless² appear to agree with Osler in this respect. Rosenow and Dunlap³ reported an epidemic of streptococcus appendicitis at Camp Culver, and according to their report the cause was a hemolytic streptococcus. Takaki⁴ and Ungerman⁵ reported the isolation of *Streptococcus pyogenes* from the vermiform appendix. Others have reported the occurrence of appendicitis due to streptococci, but it is not clear whether the organisms were hemolytic or nonhemolytic. In view of the foregoing facts, I undertook an investigation of this lesion with the object of determining the frequency with which hemolytic streptococci are found in the normal appendix and in acute and chronic appendicitis and, if possible, the rôle they play as a primary etiologic factor in appendicitis.

TECHNIC

In all, 175 appendixes were examined; the first 50 were used in a preliminary way in order to develop the technic. The appendixes were gathered from various clinics and I am indebted to Dr. Meyer and Dr. Stangl of Cook County Hospital, Chicago; Dr. Ochsner and Dr. Nuzum of Augustana Hospital, Chicago; and to others for material used. Immediately after removal and while still free from external contamination, the appendix was placed in sterile cheese cloth several layers thick, the whole wrapped in clean waxed paper and placed in the icebox. No appendix was used that had been in the icebox more than 24-36 hours; the gross examination, smears of contents and of the mucosa, and bacterial cultures of the mucosa and wall were made as soon as possible. The instruments used in dissecting the material, which was done on a sterile porcelain plate, were thoroughly sterilized before used. Cultures of the mucosa and wall were made by scraping the mucosa and muscular layers with a sterile knife, and using some of the finely divided tissue

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¹ Principles and Practice of Medicine, 1918.

² Manual of Surgery, 1919.

³ Jour. Infect. Dis., 1916, 18, p. 383.

⁴ Sei-i-Kwai, Med. Jour., 1915, 34, p. 21.

⁵ Centralbl. f. Bakteriol., 1909, 50, p. 513.

for broth and poured blood-agar plates. The plates and broth cultures were incubated for 24 hours at 37 C. and the colonies and growth in broth were then examined by methylene blue and Gram's stains. The plates were examined with special reference to hemolysis, and to the size, shape, color, elevation, pigmentation, moisture, predominance, and variety of any colonies present. Only those colonies that gave a distinct hemolysis about a small pinpoint grayish slightly elevated growth, and that on staining showed gram-positive cocci growing in chains or as diplococci, were tabulated as hemolytic streptococci. Although these tests are practically conclusive, later tests were also made and the organisms grouped in accordance with Holman's⁶ scheme. All nonhemolytic streptococci producing a green halo were classed tentatively as *Streptococcus viridans* without further attempt at classification. After cultures and smears had been made, the specimen was again placed in the icebox so as to be available for reexamination should uncertain results appear.

In this series, 48 were normal and 77 pathologic. The criteria for this differentiation will be discussed later. Two strains of hemolytic streptococci were isolated from the 48 apparently normal appendixes; these were strains 18 and 54 and belong to the type *Streptococcus infrequens* (Holman). Four strains of hemolytic streptococci were found in the 77 pathologic appendixes, namely, strains 37, 39, 71 and 104, all belonging to the type *Streptococcus infrequens*, except strain 104, which belonged to the type *Streptococcus hemolyticus* II. From this it appears that hemolytic streptococci occurred in this series in normal appendixes in 4.17 % and in the pathologic in 5.2 %. No hemolytic streptococci were isolated from 25 appendixes presenting evidence of chronic inflammation. The instances of acute appendicitis yielding hemolytic streptococci presented either ulcerative or gangrenous appendixes.

Other findings were: 108 strains of nonhemolytic colon bacilli, of which 45 strains were found in 48 normal appendixes and 63 strains in 77 pathologic appendixes; 51 strains of hemolytic colon bacillus, of which 19 were found in 48 normal appendixes; 32 strains of *Streptococcus viridans* were isolated from 48 normal appendixes. Two probable pneumococcus strains, one case of pinworm and many large unidentified bacilli, which were, no doubt, nonpathogenic, were the other results.

In the normal appendix, hemolytic streptococci, when found, occurred only in small numbers. One loopful of the macerated mucosa and wall when added to blood agar, plated and incubated for 24 hours gave 6 to 10 colonies. Streptococci in chains of 4 to 12 were found

⁶ Jour. Med. Res., 1916, 34, p. 377.

in smears from the walls and contents, but these no doubt were practically all viridans as indicated by the blood-agar plates. Leukocytes were seen only occasionally in the normal appendix. Hemolytic streptococci when isolated from pathologic appendixes were present in large numbers; furthermore, they were in almost pure culture. One loopful of the contents or macerated walls of the appendix when placed in 5 c c of blood agar, plated and incubated for 24 hours gave innumerable typical hemolytic colonies. The third dilution was usually necessary for the isolation of individual colonies. Smears from the contents and walls showed an almost pure culture of streptococci, which were gram-positive and in chains of 4 to 15. One case gave practically a pure culture of diplococci, which on growth in beef broth developed chains of 6 to 18. The smears, furthermore, showed an enormous amount of polymorphonuclear leukocytic infiltration; many pus cells and cells with ingested bacteria were present. Blood cells were present, too, but most of these were already disintegrated.

The hemolytic streptococci in this series were pathogenic for rabbits. The strains were incubated in 5 c c of plain beef broth at 37 C. for 18 to 24 hours; 3 c c were injected intravenously into the lateral vein of the ear of young healthy rabbits weighing 1,000 to 1,200 gm. Two rabbits were similarly injected with the sterile beef broth for controls. Strains 37, 39, 71 and 104 killed the rabbits in 48 to 72 hours. The organisms were recovered in pure cultures from the heart blood, 10 drops of the blood when plated giving 20 to 30 colonies. Strains 18 and 54 killed rabbits in 5 days when similarly injected. The organism was recovered from the grayish pus in the joints. No other gross lesions were noted.

As stated, of the 125 appendixes of which a record was kept, 77 were pathologic and 48 normal. This classification was made on the basis of gross appearances and clinical diagnosis. It is to be emphasized that at times it becomes extremely difficult to determine whether an appendix is normal or slightly pathologic. The statement has been made by pathologists and surgeons that in adults an absolutely normal appendix does not exist.

A word should be said in regard to the possible avenues by which hemolytic streptococci reach the appendix. As elsewhere, three routes are usually considered: contiguity, progression and hematogenous or lymphogenous channels. There was no periappendiceal involvement in any case in which the hemolytic streptococcus was found, and for

that reason, one may, with reasonable certainty, say that the mode of entrance was either by progression along the gastro-intestinal tract or hematogenous. Rosenow⁷ and others⁸ have laid stress on the hematogenous route, and name as the primary source the tonsils in a majority of the cases. Rosenow produced appendicitis experimentally by intravenous injection of streptococci and colon bacilli. A number of investigators have emphasized the selective action of certain bacteria for the appendix.

Hemolytic streptococci, as a rule, do not frequent the gastro-intestinal tract. The countless numbers of these bacteria that pass down the esophagus encounter their fate in the stomach because the ferments and acid there show a decided antagonistic action toward them. They may, however, enter the intestinal tract in a lump or mass of food. An achylia may permit them to pass into the bowel where a more favorable medium is afforded, although not the optimum. Davis¹⁰ fed hemolytic streptococci to rabbits every day for a month and only occasionally recovered them in the stools. Holman¹¹ isolated 4 strains from feces; Oppenheim 5 strains from 15 stools;¹² and Broadhurst¹³ 9 from 31 stools. This is of interest here because 5 out of the 6 strains isolated by me from normal and pathologic appendixes were of the same type.

In 1890 Kruse and Pasquale¹⁴ found streptococci in large numbers in feces of patients with acute dysentery. They were probably non-hemolytic. Beck,¹⁵ in 1892, isolated a streptococcus from the stools of cholera nostras and concluded that this organism was the causative agent, but he does not state whether the organism isolated by him was hemolytic or nonhemolytic. Lameris and Harreveld¹⁶ obtained streptococci from stools of patients suffering from diarrhea following the use of contaminated milk, but they were not pathogenic for animals.

SUMMARY AND CONCLUSIONS

Hemolytic streptococci were found in two of 48 normal appendixes (4.17 %) and in 4 of 77 pathologic appendixes (5.2 %).

⁷ Jour. Infect. Dis., 1915, 16, p. 240.

⁸ McCoy, Lancet-Clin., 1916, 116, p. 49.

⁹ Pilot and Davis, Jour. Infect. Dis., 1919, 24, p. 386.

¹⁰ Davis, Jour. Am. Med. Assn., 1919, 72, p. 323.

¹¹ Jour. Am. Med. Assn., 1919, 72, p. 319.

¹² Jour. Med. Res., 1916, 34, p. 377.

¹³ Jour. Infect. Dis., 1920, 26, p. 117.

¹⁴ Jour. Infect. Dis., 1915, 17, p. 277; Ztschr. f. Hyg. u. Infektionskr., 1893, 16, p. 1.

¹⁵ Centralbl. f. Bakteriologie, 1892, 12, p. 632.

¹⁶ Ztschr. f. Fleisch. u. Milch Hyg., 1901, 11, p. 114.

In the pathologic series they were found in acute cases only. They were not recovered from the chronically inflamed appendixes.

When hemolytic streptococci occurred in pathologic appendixes they were present in large numbers and in practically pure culture; while when found in the normal appendixes they were few in number.

Two types were found, namely, *Streptococcus infrequens* (5 strains) and *Streptococcus hemolyticus* II (1 strain).

Hemolytic streptococci apparently do not play an important rôle in the production of appendicitis; however, when they occur in the pathologic appendix, they usually predominate and appear to be the principal etiologic agent.

INFLUENZA STUDIES

II. A SEARCH FOR OBLIGATE ANAEROBES IN RESPIRATORY INFECTIONS. AN ANAEROBIC MICROCOCCUS *

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This is a report of an investigation carried out in connection with the work on respiratory diseases at the University of Chicago during the late winter and spring of 1920.

Cultures were made of 33 specimens taken from 25 persons suffering from inflammatory conditions of the upper respiratory tract, chiefly rhinitis, simple or complicated with pharyngitis, tonsillitis, or bronchitis. Six of the specimens came from patients supposed to have become ill with influenza from 4 to 9 days previously, but there is unsatisfactory clinical proof of influenza in all these cases. Rhinitis cases were selected especially for this study in order, if possible, to confirm the interesting observations of Tunncliffe on an anaerobic spirochete¹ cultivable from infections of the accessory sinuses, and on *B. rhinitis*.² The fixation experiments of Howell³ seemed to support the idea of etiologic relationship in those cases in which the latter organism was found. Tunncliffe also found an obligate anaerobe, nonpathogenic for guinea-pigs and of doubtful identity, in a case of chronic bronchitis.⁴

This paper has nothing to do with a separate study of the possibility of cultivating a filtrable virus by anaerobic means from the same materials, which is considered in another article.

The material used for cultures consisted generally of nasal washings in 40 c.c. of Ringer's solution, but in a few cases sputum and swabs were utilized. An effort was made to secure the specimens as soon as possible after the onset of the "cold," but it was often several days

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

¹ J. Infect. Dis., 1913, 13, p. 280.

² Ibid., p. 283; 1915, 16, p. 493.

³ Ibid., p. 456.

⁴ Ibid., 1913, 13, p. 289.

before the patients, mostly students, presented themselves for examination. Cultures were made at once after the specimen was secured.

No attempt was made in the beginning to duplicate exactly the technic of Tunncliffe in view of certain fallacies that are believed to attend all surface methods of isolation when applied to obligate anaerobes. The argument concerning this matter has been presented in another place.* Suffice it to say, that my preference, after extensive experience in the use of a modified Wright's method of surface isolation of obligate anaerobes, is for deep glucose agar. Dilutions of the original material without enrichment were made so as to provide well separated colonies in the depths. The tubes were broken open usually after from 2 to 3 days' incubation at 37 C., the agar pushed out into a sterile Petri dish, sliced with a boiled knife, seared with a hot spatula, and the underlying colony removed entire to freshly boiled and cooled deep brain medium. No colonies were picked from within 2 cm. of the upper surface of the agar. As a rule, three colonies were transferred, except when a variety of form indicated a larger number of bacterial types. Incubation of such transferred colonies usually resulted in vigorous growth within 24 hours, but in a few cases no growth could be secured. These may have been organisms for which the cultural conditions were unsuitable, yet the most fastidious of the sporulating anaerobes develop well under such circumstances. When growth became apparent, either by turbidity or gas production or both, a transfer on the surface of an agar or blood-agar slant for incubation under aerobic conditions was made. By this means all the cultures isolated during the early part of the investigation were shown to be facultative aerobes. Their further identification was then effected in the usual way.

When it became apparent that no obligate anaerobes could be recovered by the method of deep colony isolation steps were taken to duplicate the technic of Tunncliffe in all its details. We were unable to secure goat blood, however, and had to substitute in various experiments that of horse, sheep or rabbit. The agar used was slightly alkaline to phenolphthalein as in her procedure, although as a rule, agar to which blood is added should be adjusted more nearly to the neutral point, i. e., P_H 7, in order to avoid spontaneous hemolysis. Streaked in the usual way for isolation the blood agar slants were rendered anaerobic (degree of anaerobiosis uncertain and probably variable) by pushing in the absorbent cotton stoppers, loading with pyrogalllic acid, adding 1-2 cc of 10% lye and stoppering with a

* J. Infect. Dis., 1920, 27, p. 577.

rubber stopper. The cultures so prepared were incubated at 37 C. in an inverted position to avoid soiling the slant with lye. Open as this procedure is to objections, it provides one of the best of the surface culture methods for anaerobes; this method happened to be one with which I was thoroughly familiar. The latter part of the investigation thus involved both deep and surface methods of isolation.

In addition to the difference in cultural methods used at first, it had been my practice to pick the colonies earlier than was Tunnicliff's custom. A number of the cultures, both in deep glucose agar and on blood agar, were allowed to incubate for periods varying from 5 days to a month. In only 3 cases with deep glucose agar did new colonies appear after 72 hours, and these all turned out to be hay bacilli; with blood-agar surface cultures a tardily growing obligate anaerobe was isolated, but it proved to be a coccus. With both deep glucose agar and blood agar incubated so long, a large proportion of the picked colonies refused to grow in subcultures.

Not only were the subcultures studied carefully; in every case in which the surface culture method of isolation was used, a microscopic examination of a slide from the mixed portion of growth stained by dilute carbol fuchsin for organisms similar in morphology to those described by Tunnicliff, was made. None was found.

Even with the above close approximation to the technic of Tunnicliff, none of the cultivated forms resembled those described by her; all save one of the isolated forms were facultative aerobes. The summarized data showing the incidence of the facultative aerobes isolated from anaerobic cultures are displayed in table 1.

The rather prominent incidence of various gram-negative nonsporulating rods is emphasized. The possibility of relating some of these to the organisms, found in empyema by Gordon,⁵ was considered by him in his work in this laboratory, but with negative results except in a single instance. The frequency of *B. coli* is partly attributable to a single case (C 627) examined on four different occasions by the deep culture method, which yielded this organism each time. This was a supposed case of influenza complicated by rhinitis although there was no leukopenia; the result of the four examinations is set forth in detail:

4th day of disease.....*B. coli*, streptococcus.
5th day.....*B. coli*.
11th day.....*B. coli*, *Staphylococcus albus*.
12th day.....*B. coli*, *Streptococcus*, *Staphylococcus albus*, hay bacillus,
unidentified coliform rod.

⁵ J. Infect. Dis., 1920, 26, p. 29.

TABLE 1
SPECIMENS FROM CASES OF RESPIRATORY DISEASE YIELDING FACULTATIVE AEROBES
FROM ANAEROBIC CULTURES

	Deep Culture Isola- tion Only	Surface Culture Isola- tion Only	Both Deep and Surface Isolation			Total
Specimens.....	20	7	6			33
			Deep Only	Surface Only	Both	
Initial failure.....	1	2	—	—	—	3
<i>B. proteus</i>	2	—	—	—	—	2
<i>B. coli</i>	6	—	—	—	—	6
Intermediate*.....	3	1	—	—	—	4
Unidentified coliform.....	1	—	—	—	—	1
Hay bacillus.....	3	—	—	—	—	3
Diphtheroid.....	3	1	—	—	—	4
Gram negative diplococci.....	—	1	1	—	—	1
<i>M. tetragena</i>	1	—	—	—	—	1
<i>Streptococcus</i>	5	3	1	2	1	12
<i>Staphylococcus albus</i>	4	1	3	1	3	12
<i>Staphylococcus aureus</i>	1	—	—	1	—	2

* Gram negative rods, nonsporulating, fermenting glucose, but having no digestive action on lactose and gelatin.

An Anaerobic Micrococcus.—In one instance, a 48-hour rhinitis (C 381), an obligate anaerobic coccus, was recovered and isolated. It was associated with an unidentified coliform rod and with *Staphylococcus albus*. It was isolated from the surface of a blood-agar slant cultivated at 37 C., in which anaerobiosis was secured by the modified Wright method described. After four days' incubation, only one large white colony was present on the surface of the slant; it proved later to be *Staphylococcus albus*. On the sixth day, several tiny pin-point colonies in addition were present; they were picked to deep brain medium. The deep glucose agar in this case was markedly split by gas on the second day of incubation, and on the fourth the tube was broken open for the isolation of the well separated colonies in the agar; they were all found to be the coliform rod referred to. The slow growing nature of the coccus, since proved to develop well in deep glucose agar, precluded its isolation from the deep agar, under the circumstances.

The deep brain cultures of the picked colonies were incubated at 37 C. for several days. Growth was doubtful at first, but after a time became evident by the turbidity of the supernatant liquid. Staining was unsatisfactory from brain medium because of the natural detritus present and the small size of the organisms. Subcultures to plain agar and blood agar incubated aerobically have failed consistently to show any growth. It may be remarked that in this investigation and elsewhere I have frequently recovered anaerobically streptococci that failed to develop aerobically in the primary culture but did so in subsequent cultures. Numerous subcultures of the present organism have

been secured in brain medium, in the constricted tube with marble seal using broth with and without glucose, on blood agar under pyrogallic acid and alkali, and in the depths of glucose agar, but never on the same medium under aerobic cultivation.

The morphology is best seen in broth cultures. The organism is a minute, nonmotile coccus, occurring singly, in pairs, and in small clumps. Chains were not observed. It is gram-positive and stains readily with dilute carbol fuchsin but not so well with Loeffler's alkaline methylene blue. It is not acid fast.

Growth in all mediums is somewhat slow and distinctly better at 37 C. than at room temperature. In peptone-meat infusion broth in the constricted tube with marble seal⁶ a slight homogeneous turbidity appears below the marble in 24 hours at 37 C.; in 48 hours the broth is likely to be distinctly turbid above and below the seal. In such a medium, with Armour's peptone, which I have used simultaneously for demonstrating indol production by *B. tetani* and certain other obligate anaerobes, no indol is produced by this organism in four days' incubation. The presence of glucose in broth induces a somewhat more rapid growth. No gas is formed, but glucose is fermented with the liberation of acid. The fermentation of other carbohydrates has not been tested.

Growth in deep brain medium yields neither gas nor discoloration of the tissue.

No growth could be secured in a few trials on plain and glucose agar slants under alkaline-pyrogallol. Blood-agar slants by this method yield a delicate spreading film almost invisible, or minute bead-like colonies. Such growth suspends readily in salt solution.

Uniform heavy seeding of melted deep glucose 1 per cent. agar gives in 48 to 96 hours a band of fine colonies about 1 cm. from the surface. Longer incubation usually thickens this band from about 1 mm. to 3 or 4 mm. and often duplicates it with a thinner one a little deeper in the medium, suggesting Liesegang's rings. Fairly well separated colonies have been observed to develop in the depths of such heavily seeded cultures after a week or ten days. While these observations are by no means unique in the author's experience, such an occurrence is not common. There is no evidence in this instance that it is in any way dependent on impurity of the culture, which might be a reasonable speculation in the case of the less rare sporulating anaerobic rods. No gas is formed in glucose agar.

Light seeding of deep glucose agar seems not to predispose to ring formation to the same degree. Well separated colonies appear in the depths in 2 or 3 days, at first minute, but gradually increasing in size up to a millimeter at the end of a week. They are all dense, compact and opaque; some are flat with a leaf-like projection from the middle of one side; some are like 3 blades joined at equal angles of 120 degrees, and some are formed of 4 blades, giving the appearance of a cross when looked at from the proper direction. These variations appear not to have any special significance. Subcultures from isolated colonies show the same variations in colony form. This anaerobe appears not to have any action on milk. No growth could be secured in gelatin.

There is no evidence of pathogenicity for guinea-pigs or rabbits.

A guinea-pig, weighing 240 gm., was injected with 1 cc of a 4-day glucose-broth culture prepared in the constricted tube with marble seal. There was no result during a week's observation, at the end of which time the animal had gained 26 gm. in weight.

A rabbit, weighing 2500 gm., was injected intravenously with 1 cc of a 5-day plain broth culture from a constricted tube. There was no harmful effect on this animal.

⁶ Hall: Univ. Calif. Public. in Path., 1915, 2, p. 147.

A normal rabbit, weighing 1850 gm., and a guinea-pig, weighing 290 gm., were each perfused with 1 c.c. of a 3-day glucose broth culture in the right nasal passage. No symptoms of discomfort or respiratory disease appeared during the week immediately following.

The cultures used were in every case active vigorous suspensions of moderate turbidity.

Obligately anaerobic cocci have not been described often. Jungano and Distaso⁷ quote some references, but the writer has no access to the work of these authors at present. Wehrsing and Marwedel⁸ found an obligate anaerobic streptococcus forming gas (!!) in a war wound. It was described as blackening blood broth and presenting a putrid odor, and was thought to be identical with *Streptococcus putridus* of Schottmüller. Weinberg and Seguin⁹ consider that Wehrsing and Marwedel probably had to do with a mixed culture containing some gas forming anaerobe difficult of isolation. This supposition is supported by the fact of gas production which would be unusual for a coccus.

It is impossible to assign our organism definitely at this time. Morphologically, it resembles the staphylococci; culturally, it resembles the streptococci.

SUMMARY

No organisms resembling those described by Tummicliiff were seen in the cultures examined in this series. The results seem more comparable to those recorded by Norton,¹⁰ who failed to find any obligate anaerobes in the cultivation by anaerobic methods of sputum, nasopharyngeal swabs, and blood from influenza patients.

The probability of obligate anaerobes developing in the respiratory tract seems slight in view of the excessive air supply, and the above findings indicate that such infections occurred, if at all, only infrequently in the cases of respiratory disease presented for examination during the winter and spring season of 1920.

There is nothing to show that the obligate anaerobic coccus found in one instance was pathogenic.

⁷ *Les Anaerobies*, 1910.

⁸ *München. med. Wchnschr.*, 1915, 62, p. 1023.

⁹ *La gangrène gazeuse*, 1918.

¹⁰ *Am. Jour. Public Health*, 1919, 9, p. 593.

GONOCOCCUS TYPES. I

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During the past decade and a half a large amount of work has been applied to typing the strains of various pathogenic organisms. Not only has the "colon-typhoid-dysentery group" received its share of attention, but, more recently, the pneumococcus, the meningococcus and the streptococcus groups have been studied. Torrey¹ published results on typing of gonococci by agglutination and absorption and Torrey and Teague² results of complement fixation as a means of typing. On the basis of his first work Torrey came to the conclusion, that if the principle that specific relationships are based on the absorption of the major agglutinins then the gonococci include a large number of organisms which differ among themselves as greatly as the different types of dysentery bacilli and streptococci.

Despite the fact that he seemed to doubt the validity of this principle, nevertheless, he believed that the gonococcus group is composed of heterogeneous types of which three are very distinct. Six of his ten strains belonged to these three types. The relationships of the remaining four were not clear. The result of the complement fixation work made him more skeptical. Here type 2 could not be differentiated from type 1; thus giving only two distinct types. He was not quite sure whether the types were radically different as in the dysentery family, or only slight variations of the family type due to modifications of receptors. Still he continued to speak of them as forming heterogeneous groups (types). Watabiki³ found that by complement fixation the eight strains he worked with could be separated into two different groups. He was sure that the differences between them were not as distinct as those of the dysentery group. "The differences among the gonococci were only comparative and not distinctive," he said.

Louise Pearce⁴ studied the relation between gonococci causing adult male urethritis and those of vulvovaginitis in small girls. She

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¹ Jour. Med. Research, 1907, 16, p. 329.

² Jour. Med. Research, 1907, 17, p. 223.

³ Jour. Infect. Dis., 1910, 7, p. 159.

⁴ Jour. of Exp. Med., 1915, 21, p. 289.

used agglutination and fixation and thought that the immunity test offered an indication that at least a part of the clinical difference in the two infections was due to the inherent difference in the two types of organisms causing them. Yet the two types were not distinct and separate from one another, the distinction being relative only.

Dr. W. B. Wherry suggested that this problem be studied using the cultural methods which he and Wade Oliver found successful in growing gonococci, i. e., partial tension methods. During the summer of 1917 I began to collect strains of gonococci from urethritis, vulvovaginitis, and ophthalmia. The majority of these strains came from patients in the wards or dispensary of the Cincinnati General Hospital. The cases of vulvovaginitis were acute or subacute in character, from a few days to several weeks duration and in children from a couple of weeks to ten years of age. Only three were over six years of age. The cases of urethritis were acute, subacute and chronic. Some of the ophthalmias had been treated for a week or more before they came to my attention.

The partial tension method was used mainly in the isolation work. A culture of hay bacillus on slanted solid medium was attached to a slanted agar tube, inoculated with the desired organisms, by means of a piece of rubber tubing. In this way the mouths of the two cultures were brought close together, and the columns of air in both were united through the cotton plugs. If by chance, the rubber tubing was not close-fitting each end was wrapped with rubber bands. By its use abundant growths were obtained. For comparative purposes the aerobic method was also applied. It gave, however, but scanty growth at best, and frequently none at all. This method also permitted such secondary invaders as were present to grow more luxuriantly or to dominate. Even with the partial tension method diphtheroids and streptococci sometimes gave considerable trouble. As a rule, however, a pure culture of gonococci was obtained on the first or second transplant.

The medium used was an ascites agar composed of three parts of plain saltfree nutrient agar and one part of ascites fluid. Other mediums were tried, and while some of them were useful, this one was finally adopted as the simplest and most reliable under all conditions. It is interesting that gonococci prefer the saltfree material.

No marked differences in growth could be demonstrated on mediums ranging in acidity from 0.5 to 1.5 (phenolphthalein), and an approx-

imate mean of 0.9 acid to phenolphthalein corresponding roughly to a neutral reaction to litmus was adopted. Rabbit-blood-agar was found an especially good medium. For isolation, however, it had the disadvantage of being too opaque, and for stock cultures it permitted a too rapid dying-out of the cultures. The organisms growing luxuriantly at first died in the succeeding period. The cultures were kept in the incubator for not more than 16-20 hours and remained at room temperature, until they were transplanted, i. e., 8 to 10 days. Some strains appeared to be more viable and resistant than others, living for weeks or even months without transplanting. Whenever a culture remained alive for several months it was due to secondary superimposed colonies upon the old diffuse growth, starting as small elevated dots growing larger and larger till such a colony sometimes measured from 4-6 mm. in diameter and had when very big and fat a faint yellowish pink coloration. On seeing these big colonies one can be quite sure that the organism is alive no matter how long it has grown since the last transplant. Recently I found that a strain which I had given up as lost four months ago was still living in a capped tube that had been put aside. Yet that strain was a very sensitive and delicate grower. Some strains grow very well for a long time, and then for some reason become sensitive and are propagated with difficulty. In such cases it may be necessary to attend to the cultures very carefully. Sometimes the colonies are so small that no apparent growth is visible, yet on spreading and reinoculation the culture may prove successful.

Having secured several representative strains of the three different types of infection, rabbits were injected with them. For the weekly injections suspensions of living organisms were used and these were given intravenously and intraperitoneally in increasing doses up to an amount representing the growth of 4-6 slant cultures. It was found that by using the intraperitoneal method fewer animals succumbed before a high titer serum was obtained. As it was, many of the animals lost weight at first and even became very emaciated, but most of them picked up and afterwards were able to withstand large doses. From time to time, the potency of the serum was tested, and when a serum of sufficiently high titer was found, from 25-30 c c of blood was taken from the heart with a sterile syringe. After bleeding the animal was allowed to rest several weeks before injections were again resumed. Some rabbits responded more quickly than others and produced a potent serum after a month or more, others responded rather slowly.

The former finally emaciated and eventually died of some intercurrent infection. Nevertheless, it was possible to bleed them once or twice before they died. The latter generally produced a comparatively low titer serum and seemed to be unaffected by the treatments.

In addition to the 49 strains obtained directly from the clinical cases I had at my disposal 38 strains sent to me through the courtesy of several laboratories, including the so-called Torrey strains.

Fermentation tests were applied to most of the strains. Practically none of them fermented maltose. Yet two seemed to reveal a slight initial acid production indicated by a faint acidity, which, however, after 48 hours was rendered alkaline. All of them formed acid on dextrose, some actively, others slightly, and only in one instance was the reaction doubtful. All of the fermentation tests were made under partial tension on agar slants. After 24 hours the tubes were separated and the final readings recorded 24 hours later.

For absorption experiments large tubes (15 x 1.7 cm.) were used. The slant occupied two thirds of the tube and thus gave a large surface. Preceding the inoculation of the medium the water of condensation and hysteresis was removed with a pipette.

In order to obtain clear-cut and reliable results the serum had to be properly diluted. For this purpose 1 c c of various dilutions of the serum was absorbed by the growth of one large slant of the homologous strain. The lowest that could be used was that dilution which after absorption revealed complete absence of agglutinins when tested with the absorbing strain. The absorption by the producing strain being generally more complete than that of the related ones, the dilution for the actual work had to be somewhat higher. A 24 hour growth was washed off with 1 c c of the diluted serum, placed in small heavy walled tubes, corked, incubated for 4-6 hours at 55 C. and left over night in the ice chest. The following morning the tubes were centrifuged and the clear fluid used for the tests. For each strain a row of small test tubes was set up in a wooden block with holes. The tubes were about 3 cm. long and had a capacity of 2.5-3 c c. To all the tubes but the first in each row 0.5 c c of salt solution was added. Then the absorbed serum was placed by means of a calibrated pipette so that the first empty tube received 0.3 c c, and the second 0.5 c c. The contents of this tube were mixed and from it 0.5 c c transferred to the third tube and the process repeated until the desired final dilution was reached. In the meantime a necessary amount of a suspension of the strain of

gonococci that had been used in producing the serum was prepared. And 0.5 c.c. of it was added to all the tubes but the first of each set, which received only 0.3 c.c. Thus each serum dilution was rediluted 1-1. So that when the serum dilution used for absorption was 1-250, the final dilutions ranged 1-500, 1-1,000, etc. The contents of the tubes were thoroughly mixed by pipetting, corked, and the whole outfit placed in a small incubator at 50-56 C. over night. Usually a preliminary reading was made after five to six hours, but the final one was made the following morning, and the results recorded as follows: +++ mean complete agglutination, i. e., all the organisms were gathered into large flakes and had settled in the bottom of the tube, leaving a clear supernatant fluid; ++ indicate that something like half of the flocculi were not well developed, and remained suspended in the fluid; + means a distinctly visible flocculation of the cells, the clumps being of a comparatively small size so that most of them remained suspended; 0 means absence of agglutination. The estimations were made entirely with the naked eye.

TABLE 1

STRAIN 1 SERUM, 1 C.C. OF 1-250 DILUTION, WAS ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 1

Gonococcal Strains	Serum Dilutions				Immuno- logic Types
	1:1000	1:4000	1:8000	1:16000	
1-4, 6-9, 12-17, 24.....	0	0	0	0	1
27, 30, 31, 35, 39, 40, 41.....	0	0	0	0	1
5, 10, 11, 18-23, 25, 26.....	+	0	0	0	1
28, 29, 32-34, 36-38.....	+	0	0	0	1
42, 45, 47, 54, 58, 63, 64, 68.....	+++	+++	+++	+++	2
46, 50, 52, 55-57, 59, 60, 62.....	+++	+++	+++	++	2
69, 70, 74-76, 78.....	+++	+++	+++	++	2
43, 44, 48, 49, 53, 61.....	+++	+++	+++	+	2
66, 67, 71, 72, 77.....	+++	+++	++	+	2
79, 80, 81.....	+++	+++	++	+	3
82, 83.....	+++	+++	+++	++	4
84.....	+++	+++	++	+	5
85.....	+++	+++	+++	++	6
Control.....	+++	+++	+++	++	
Control.....	+++	+++	++	+	

Table 1 shows the absorption results of the serum of a rabbit injected with strain 1, isolated from an ulcerative, aortic endocarditic lesion in a young colored man who had acute urethritis previous to entering the hospital. This serum was diluted 1-250 and 1 c.c. of this dilution absorbed by the growths of one large slant each of the various strains, and then tested with strain 1. Forty-one strains either completely absorbed the agglutinins or left only traces. Hence the absorbing strains must be closely related to the strain used to produce the

serum, and therefore they are placed in a group which is called type 1. None of the other strains were able to remove any of the agglutinins incited by this strain. In a dilution of 1-200 similar facts were brought out. In the rearrangement of the tables the more decisive dilutions only were used. Strains giving identical results were handled collectively. The heavy type indicates that after absorption with these strains enough agglutinins were present to cause a partial, i. e., + agglutination of the immunizing strains in a higher dilution than that recorded. The variations in the higher dilutions should not be taken as an index of a slight binding capacity by some of the strains. It happened as the controls show, that the agglutinability of the same strain varied somewhat from day to day and at times this variability was quite marked, and more pronounced in some strains than in others. Often a whole set including the controls on a certain day agglutinated much lower than usually. Now a serum could not be absorbed with all the strains at one time. About twenty strains were the most that could be handled in a day. They were selected at random, but usually included the representatives of at least two types. The records of these daily experiments formed the bases for compilation of the final tables, which were rearranged according to the serological types. Slight differences in the higher dilutions were present even in the daily tests and were due to the nature of the technic employed. Since each strain was handled individually a little difference in the amount of the serum used for washing off the growth, combined with the following separate dilutions of the absorbed serums, made the higher dilutions vary in concentration. The slight variation in the amount of growth used had little influence on the results, as the growth of a large and ordinary slant gave practically identical results. As a rule the agglutination of the immunizing strains after absorption by the heterologous non-absorbing strains was just as good or even better than with the unabsorbed serum used as a control.

Identical results were obtained with serums produced by rabbits injected with strains 2 and 7. These serums were of nearly the same potency; they were diluted 1-200, absorbed by the growth of one large slant of each of the various strains and tested with the homologous strains used in immunizing the animals. Their agglutinins were bound only by those strains that had absorbed agglutinins from strain 1 serum. Similar facts were brought out even when these three serums were diluted 1-100. With this dilution only the homologous inciting strains

were able to bind the agglutinins completely, the other type 1 strains generally left moderate amounts unabsorbed.

With one group of strains disposed of the next problem was to type the others. Table 2 gives the results of absorption with the serum induced by strain 63. The serum was diluted 1-100 and 1 c c absorbed by the growth of one large slant each of the various strains and then reagglutinated with strain 63. It will be seen that none of the members of type 1 were able to absorb the agglutinins of this serum altho many other strains absorbed them completely. These are just as unmistakably related and form another type. This was named type 2. The binding capacity of strains 67, 69-74 varied, some of them at times were able to absorb the agglutinins completely, but on the average they left traces unbound. Strains 75 and 76 only partially bound the agglutinins; and 77 and 78 left them always completely intact. Type 2 is composed of four distinct races, indicated by the letters "a-b-c-d." Their relation in type 2 will be discussed later. Type 2a strains absorbed the agglutinins in a 1-50 dilution, the heterologous strains left them intact even in 1-200 dilution. Similar results were obtained with a serum produced by injection with strain 42. The binding by type 2a strains was complete in a 1-20 while strains of the other types failed entirely even in a 1-100 dilution.

TABLE 2

STRAIN 63 SERUM, 1 c c OF 1-100 DILUTION, WAS ABSORBED BY THE GROWTH OF ONE LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 63

Gonococcal Strains	Serum Dilutions				Immuno- logic Types
	1:200	1:400	1:800	1:1600	
1, 8, 18-20, 22, 32, 33, 36, 38.....	+++	+++	+++	+++	1
2, 4, 5, 14, 21, 29-31, 40.....	+++	+++	+++	++	1
7, 11, 24, 39, 41.....	+++	+++	+++	+	1
3, 9, 10, 12, 23, 25, 28.....	+++	+++	++	+	1
26, 27	+++	+++	+++	0	1
42-66	0	0	0	0	2a
67, 69-74	++	0	0	0	2b and c
75, 76	+++	++	0	0	2d
77, 78	+++	+++	+++	+++	2d
79, 80, 81.....	+++	+++	+++	++	3
82, 83	+++	+++	++	0	4
84	+++	+++	+++	+++	5
85	+++	+++	+++	+	6
Control	+++	+++	+++	+++	
Control	+++	+++	++	0	

Seven strains were unable to remove any of the agglutinins of the serums incited by strains of the two types. Table 3 gives the absorption results of the serum produced by injections with strain 81. The

agglutinins were bound completely by strains 81, 80 and 79. Identical facts were disclosed with a serum produced by strain 79, in a dilution of 1-100. Their relationship is clear and they form type 3.

TABLE 3

STRAIN 81 SERUM, 1 CC OF 1-150 DILUTION, WAS ABSORBED BY THE GROWTH OF 1 LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 81

Gonococcal Strains	Serum Dilutions				Immuno- logic Types
	1:300	1:1200	1:2400	1:4800	
3, 5, 15 32, 33.....	+++	+++	+++	++	1
1, 2, 7, 11, 14, 16.....	+++	+++	+++	+	1
39, 36, 40, 41.....	+++	+++	+++	+	1
45, 51, 53, 54, 63, 70, 77.....	+++	+++	+++	+++	2
49, 65, 67, 69, 73.....	+++	+++	+++	++	2
42, 44, 50, 57, 66, 71, 74, 76.....	+++	+++	+++	+	2
46, 48, 64, 68.....	+++	+++	+++	0	2
81, 80, 79	0	0	0	0	3
83	+++	+++	+++	+++	4
84	+++	+++	+++	0	5
85	+++	+++	+++	+++	6
Control	+++	+++	+++	+	

Table 4 shows the absorption results of strain 82 serum. Its agglutinins were removed by strains 82 and 83 only. Strain 82 absorbed them even in 1-20 dilution and the absorption by strain 83 was always complete on five repeated tests, and once with half of the normal amount. These two related strains form type 4.

TABLE 4

STRAIN 82 SERUM, 1 CC OF 1-100 DILUTION, WAS ABSORBED BY THE GROWTH OF 1 LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 82

Gonococcal Strains	Serum Dilutions				Immuno- logic Types
	1:200	1:400	1:800	1:1600	
8, 16, 17, 19, 20, 32, 33, 36.....	+++	+++	+++	+++	1
1, 5, 7, 12, 14, 18, 34, 39.....	+++	+++	+++	++	1
2, 4, 15, 21, 22, 38, 40.....	+++	+++	+++	+	1
43, 44, 48, 51, 70-72, 74, 75-78.....	+++	+++	+++	++	2
50, 53, 46, 60, 64, 67, 69.....	+++	+++	+++	+	2
55, 59, 66, 68.....	+++	+++	+	+	2
79, 80, 81	+++	+++	+++	+++	3
82, 83	0	0	0	0	4
84	+++	+++	+++	++	5
85	+++	+++	+++	+	6
Control.....	+++	+++	+++	+++	
Control	+++	+++	+++	+	

The serum produced by the injections with strain 84 differentiates this strain from the others, as shown in table 5. It alone was able to absorb its agglutinins and thus forms a type by itself, namely type 5.

TABLE 5

STRAIN 84 SERUM, 1 C.C. OF 1-150 DILUTION, WAS ABSORBED BY THE GROWTH OF 1 LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 84

Gonococcal Strains	Serum Dilutions				Immuno- logic Types
	1:300	1:1200	1:2400	1:4800	
2, 11, 40, 33.....	+++	+++	+++	+++	1
1, 7, 35, 39, 41.....	+++	+++	+++	+++	1
3, 5, 16, 17, 32.....	+++	+++	+++	+	1
44, 45, 48, 57, 64.....	+++	+++	+++	+++	2
42, 49-51, 54, 70, 72.....	+++	+++	+++	+++	2
46, 53, 63, 65, 73.....	+++	+++	+++	++	2
66, 69, 71, 76, 77.....	+++	+++	+++	0	2
79.....	+++	+++	+++	+++	3
80, 81.....	+++	+++	+++	+	3
83.....	+++	+++	+	0	4
84.....	0	0	0	0	5
85.....	+++	+++	+++	++	6
Control.....	+++	+++	+++	+++	

This leaves one strain that did not show any relation to any of the types, and the absorption experiments with the serum produced by it confirmed this. None of the strains were able to absorb its specific agglutinins in a 1-100 dilution, as shown in table 6. Similar facts were obtained in a 1-200 dilution. Strain 85, however, was able to bind them completely even in 1-10 dilution.

TABLE 6

STRAIN 85 SERUM, 1 C.C. OF 1-100 DILUTION, WAS ABSORBED BY THE GROWTH OF 1 LARGE SLANT OF THE FOLLOWING STRAINS AND TESTED WITH STRAIN 85

Gonococcal Strains	Serum Dilutions			Immuno- logic Types
	1:200	1:400	1:800	
24, 25, 27, 28, 30.....	+++	+++	+++	1
2, 4, 6, 8, 29.....	+++	+++	++	1
1, 3, 11, 16, 23, 36.....	+++	+++	++	1
7, 10, 12, 14, 18, 20.....	+++	+++	+	1
19-22, 31-34, 38.....	+++	+++	+	1
47, 57, 58, 60, 61, 63.....	+++	+++	+++	2
49, 55, 56, 64, 66.....	+++	+++	++	2
42, 46, 48, 71, 75, 76.....	+++	+++	+	2
79, 80, 81.....	+++	+++	++	3
82, 83.....	+++	++	+	4
84.....	+++	+++	+	5
85.....	0	0	0	6
Control.....	+++	+++	+++	
Control.....	+++	+++	++	

SUMMARY

The results of absorption experiments without question prove that the gonococci are a collection of organisms that falls into distinct clear-cut immunologic types, having very little relation with one another. The agglutinins of a serum produced by one type cannot be absorbed by any of the strains forming the other types no matter how highly the

serum is diluted and how much of the growth used. Thus strain 1 serum was diluted 1-250 and 1 c c absorbed by the growth of two large slants of selected heterologous strains. None of them were able to absorb any of the agglutinins. Yet the homologous strains absorbed them completely in one half and one third of that amount. The 85 strains included in this communication fell into six very distinct serologic types. By far the largest number of the strains belonged to either type 1 or 2. The Torrey strains from three laboratories established themselves as members of type 1, while of the ten strains received from another laboratory only 4 fell into this type. The remaining 6 formed the C race of type 2.* No attempt will be made to explain this difference as to types in the Torrey strains as it would lead to mere assumptions.

* Strain 66 is a member of the b race of type 2.

INFLUENZA STUDIES

III. ATTEMPTS TO CULTIVATE FILTRABLE VIRUSES FROM CASES OF INFLUENZA AND COMMON COLDS *

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During the winter of 1919-20 we attempted to cultivate filtrable viruses from certain respiratory infections, employing the technic of Foster,¹ and in addition, other methods that seemed promising. Fifty-five samples of nasopharyngeal secretions from 44 individuals have been studied, 38 of the samples being from common colds, 9 from influenza, and 8 from normal persons. Most of the colds were simple acute rhinitis, 7 were accompanied by a bronchitis, 1 by pharyngitis, 2 by sinusitis, 1 by tonsillitis, and 1 proved to be a nasal diphtheria. The time of collecting these samples after the period of onset of the disease is shown in table 1.

TABLE 1
TIME AFTER ONSET WHEN SAMPLES WERE COLLECTED

	Days							
	1	2	3	4	5	5 After	6th	
Colds	11	2	8	8	—	—	9	38
Influenza	—	1	2	2	1	1	2	9
Normal	—	—	—	—	—	—	—	—
Total	11	3	10	10	1	1	11	55

In collecting the samples, the nasopharynx was washed out with 50-75 c c of warm Ringer's solution into a sterile glass or rubber stoppered bottle containing glass beads. Five of the samples stood overnight in an icebox, but the others were handled promptly, in most cases within 2 hours of collection.

In every case a loopful of *B. prodigiosus* was added to the sample before filtration; it was then shaken thoroughly with the glass beads, and a plain agar slant made to show that the organism was present.

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago.

¹ J. Infect. Dis., 1917, 21, p. 451.

Then the sample was passed through a Mandler 6 x 1.5 cm. filter, under a vacuum pressure of from 14 to 60 cm. of mercury, the time of filtration being from 5 to 30 minutes. The freedom of the filtrate from *B. prodigiosus* and other bacteria was determined by inoculating 5 c c of it into about 20 c c of glucose broth. This test for permeability was made each time because often a previously impermeable filter will suddenly allow organisms to pass through.

The medium used most was the tissue-ascitic fluid employed by Foster¹ and by Flexner and Noguchi.² About 15 c c of ascitic fluid were put into culture tubes 20 x 1.5 cm., a piece of fresh sterile rabbit kidney was added, and a 4 cm. layer of mineral oil, autoclaved at 20 pounds for 20 minutes on 2 successive days, was poured on top. These tubes were incubated at 37 C. for several days to determine their sterility. At first the blood in the rabbit kidney laked and formed a clear rosy zone around the tissue. After a few days this faded and the pink tissue, gradually became gray.

Other mediums used were:

1. Equal parts of ascitic fluid and plain 2% agar poured into a tube over a piece of sterile rabbit kidney and covered with a layer of sterile mineral oil.
2. One part of horse serum, inactivated at 56 C. for 30 minutes to ten parts of 2% agar, with sterile rabbit kidney and a layer of oil.
3. Noguchi's³ serum water, made with horse serum, under oil.
4. Glucose broth with sterile rabbit kidney.
5. A modification of Noguchi's serum water, without the sterile tissue, and with 0.5% peptone and 1% glucose, the P_H value adjusted to 8.
6. A plain 1% glucose meat infusion broth with 2% peptone, and 0.5% NaCl.
7. Serum-glucose broth, with 1 part of horse serum to 4 of broth.
8. Ascitic-glucose broth, with 1 part of ascitic fluid to 1 of broth.

The first 4 mediums were incubated 48-72 hours to determine their sterility; the last 4 were boiled vigorously just before using to expel oxygen, and, in the case of the serum glucose broth and the ascitic glucose broth, the ascites and serum were added at the time of inoculation of filtrate. A clear mineral oil was used as the anaerobic seal

¹ J. Exper. Med., 1913, 8, p. 461.

² Ibid., 1911, 14, p. 99.

in addition to the considerable depth of fluid in the first 3 mediums above mentioned; in the last 5, anaerobiosis was secured by means of a marble seal in the constricted tube designed by one of us.⁴ The agar and glucose broth used in these mediums had a veal infusion basis, and the reaction was P_H 8.

The mediums were inoculated by means of sterile graduated pipets, with an amount of filtrate varying from 0.5 c c to 10 c c. These cultures, with the uninoculated controls accompanying them in each case, were incubated at 37 C. Not every kind of medium was used for all the samples, but each was used a number of times, as shown in table 2.

TABLE 2
DISTRIBUTION OF MEDIUMS IN 55 SAMPLES

Kind of Medium	Times Used
Ascites-kidney	44
Ascites-kidney-agar	12
Serum-kidney-agar	17
Noguchi's serum water.....	23
Glucose-kidney broth	26
Modified Noguchi's serum water.....	20
Plain glucose broth.....	48
Serum- or ascites-glucose broth.....	33

With 13 sets of cultures, a growth of *B. prodigiosus* appeared in the inoculated tubes within 72 hours, showing that the filters used were permeable to bacteria of that small size. In 3 others a quick growth of the hay bacillus, and in one of *Staph. albus*, indicated some fault of technic. The other 38 sets of cultures were incubated for from 3 to 6 weeks, and were carefully observed from day to day. In all of the ascitic-tissue tubes, after a few days' incubation, a faint haze appeared around the tissue. This usually spread slowly upward, always being sharply demarcated from the clear fluid above. Sometimes this haze was more pronounced in the inoculated tubes; often no difference could be seen between them and the uninoculated ones; and sometimes the cloudiness was more marked in the control tubes. Aerobic subcultures on blood agar and glucose broth were made from material pipetted from these hazy zones. Where the haziness was pronounced, these subcultures often showed bacterial growth, the most common organisms being *Staph. citreus*, a tiny plump gram-positive bacillus, and diphtheroid bacilli. Occasionally other cocci were found. The source of these organisms, which appeared as late contaminations

⁴ Univ. Calif. Pub. in Path., 1915, 2, p. 147.

in the tissue-ascitic fluid culture tubes, remains unexplained. The nature of the organisms, their late appearance, often after more than two weeks' incubation, and their frequent occurrence in controls as well as in inoculated tubes, all seem to indicate that they were not acquired during filtration and that they were not due to faulty technic. Perhaps they were the result of organisms deeply lodged in the kidney tissue, which required a long time to grow to the surface.

Twenty-three sets of ascitic-tissue tubes were incubated with no bacterial contamination for from 3 to 6 weeks, until discarded. Subcultures made from them into the same medium from time to time, and the controls as well, soon took on the characteristic appearance, with the sharply demarcated haze around the tissue; but those made into the solid medium, such as ascitic-tissue agar and serum-tissue agar, showed little change. A haziness in the tubes which contained no tissue always meant bacterial contamination, which could be demonstrated by strains and subcultures on agar and in broth. In all mediums containing tissue there was some haziness after a time, due apparently to autolysis of the tissue.

Giemsa, Gram, and Loeffler's methylene blue stains were made at intervals, beginning with the seventh day, from all cultures and subcultures, using material withdrawn from around the tissue with a sterile capillary pipet. With the Giemsa stains, the methods used by Foster and by Noguchi were carefully followed. The films were air-dried, fixed one hour in methyl alcohol, stained overnight in a jar containing one drop of Giemsa stain to every 1 c c of distilled water, immersed in acetone a few seconds to remove the excess stain, and then washed in distilled water and dried. With Gram's method, safranin was used as a counterstain.

Microscopically these stains from the ascitic-tissue cultures presented a puzzling picture. They showed, among the *débris*, a great many tiny round bodies of various sizes, purple with Giemsa's stain, and gram-positive. There was little uniformity or regularity about them. These bodies often strikingly resembled tiny cocci, and, while usually occurring singly, were often in pairs and groups, and occasionally in short chains. Similar bodies seemed to be equally numerous in the stains made from control tubes, and also in plain ascitic fluid. They were particularly abundant in the Gram stains, especially if the materials used had not been freshly filtered, or if the slides were not very clean.

They were found in abundance in all mediums containing autolyzed tissue. To a lesser extent they were present on stains made from the cultures and controls in the other mediums used after incubation for a time, and finally they were found on stained blank slides. There were no differences noted in the slides made from cases of colds or influenza, or from normal persons.

These bodies might have been considered the globoid bodies described by various investigators, but we were unable to convince ourselves that they were other than artefacts derived on the one hand from the disintegration of the tissues added to the mediums, or on the other, from precipitates in the mediums and stains employed. The necessity of great caution in interpreting microscopic findings from such "cultures" is well illustrated in the extraordinary retraction made recently by certain English investigators.⁵ We consider our results totally negative so far as the cultivation of a filtrable virus from our material was concerned.

In order to be sure that these negative results were not due to the unsuitability of the mediums, and that it was perhaps justifiable to assume that filtrable viruses ought to grow in them, some of each type of medium was used for the cultivation of certain strict anaerobes and especially delicate facultative aerobes. *B. sporogenes*, *B. botulinus*, *B. welchii*, *B. tetani*, *B. chauvæi*, and *Vibrio septique* all grew well during 48 hours' incubation. The freedom of these cultures from aerobic contamination was checked by a plain agar slant. *Streptococcus viridans* grew well in all of the mediums; *B. influenza* grew in all containing tissue and with special luxuriance in the tissue-ascitic fluid; *Diplococcus gonorrhœæ* grew in all except the glucose broth, and also showed a special preference for the tissue-ascitic fluid. Except in the first few trials, every new lot of medium was tested by growing all of these organisms in it.

DISCUSSION

The methods used by Foster with colds have been followed as closely as possible. Gibson, Bowman, and Conner⁶ followed Foster's technic in their work with influenza, and reported positive results. Our attempts to repeat this work have been unsuccessful. After one trial we did not attempt to employ the modified medium of Wilson,⁷ both

⁵ Brit. Med. Jour., 1919, 2, p. 233.

⁶ Brit. Med. Jour., 1918, 1, p. 645; 1919, 2, p. 331.

⁷ Ibid., 1919, 1, p. 602.

on account of the technical difficulty of preparing it without contamination, and because there was no way in which, by preliminary incubation, freedom from bacterial contamination could be assured.

Noguchi, Foster, and others stated that all samples of ascitic fluid were not suitable for the growth of the organisms which they found. The only criterion seems to have been whether or not their cultures grew in it. This test it was obviously impossible for us to apply. However, it seems that the decided preference shown by very delicate bacteria for the mediums containing samples of the ascitic fluid used, and especially for the plain ascites-tissue, is a criterion of some value. That the conditions of anaerobiosis were fulfilled is proved by the ready growth of the strict anaerobes used.

The first sample of ascitic fluid became contaminated in the laboratory and had to be filtered to insure its sterility. The samples used later were originally sterile, free from bile color, and formed a loose fibrin in the culture tubes. These three qualities Noguchi emphasized as being highly desirable, and in the mediums made with these later samples the bacteria tested grew with great luxuriance.

With the idea that perhaps a possible disadvantage of the Foster-Noguchi medium lay in the fact that there was no suitable way of removing oxygen just before use, several kinds of mediums were used that could be boiled out, using the mechanically sealed constricted tubes referred to above. These furnished ideal conditions of anaerobiosis. A possible error lay in the addition of glucose to such mediums, for no matter how carefully a reaction is adjusted, the presence of glucose is always a potential source of acid. Both Noguchi⁸ and Foster¹ stated that the medium they used was slightly alkaline, though the degree of alkalinity and the indicator used were not mentioned.

SUMMARY

These experiments offer no evidence in support of the theory that the cause of either common colds or influenza is a filtrable virus.

In attempting to cultivate filtrable viruses from the nasopharyngeal secretions in colds and influenza, no bodies were found in the "cultures" which could not be found also in those from normal persons, in controls in all simple mediums examined, and on blank slides.

⁸ Jour. Exper. Med., 1912, 16, p. 199.

It is recognized that negative experiments, limited to the attempted cultivation of a filtrable virus, and including no attempts to reproduce the disease in animals, do not offer conclusive evidence that such a virus is not involved.

No conclusions can be drawn concerning influenza, on account of the few cases examined, together with the fact that samples of such were not collected during the earliest stages of the disease. However, the uniformly negative results obtained with a large and representative number of colds are not without significance.

MERCURY COMPOUNDS IN THE CHEMOTHERAPY OF EXPERIMENTAL TUBERCULOSIS IN GUINEA-PIGS. I

STUDIES ON THE BIOCHEMISTRY AND CHEMOTHERAPY
OF TUBERCULOSIS. XXI

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Perhaps no drug has a longer history of use in therapy than has mercury. Much of this history, and especially that of the newer organic mercury compounds, has been reviewed by Schamberg, Kolmer and Raiziss¹ and by others, and need not receive further attention here. In tuberculosis, mercury in various forms and by various methods has been used to a greater or less extent clinically since the time of Paracelsus early in the sixteenth century. There have been certain periods when it was in especial favor and others when it was in disrepute. Hall,² while not regarding it as a specific, thinks that small doses of mercurial preparations are "our most potent chemical weapons against this disease." Many others at about the same period, published favorable reports on mercury therapy of tuberculosis. In 1908 and 1909 Barton L. Wright³ reported a considerable number of cases treated with mercuric succinimide and potassium iodide alternately, and his favorable reports were followed by many others, who used the same or similar treatment. The general consensus of opinion seems to be that mercurials have a good effect on the condition of the patient, but that mercury is in no sense a specific in tuberculosis.

Only one report in the literature has been found by me on the use of mercury in experimental tuberculosis in animals. George Cornet⁴ tested mercuric chloride among a series of disinfectants, in 8 guinea-pigs. He injected mercuric chloride for 10 days until the animals began to show toxic effects; he then inoculated them with tubercle bacilli. He was unable to note any difference in the extent

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¹ Am. J. Syphilis, 1917, 1, p. 1.

² Am. J. Med. Sc., 1889, 98, p. 45.

³ New York Med. Jour., 1909, 89, p. 1180.

⁴ Ztschr. f. Hyg. u. Infektionskr., 1889, 98, p. 98.

of the disease between the mercurialized animals and the controls. Some died of mercurial poisoning early, and all the others showed generalized tuberculosis.

Robert Koch⁵ stated that mercury in vapor form inhibited the growth of the tubercle bacillus in the test tube but was entirely without influence on the progress of the disease in animals.

In view of the long continued and repeatedly favorable use of mercurials in the clinical treatment of human tuberculosis and the paucity of experimental evidence as to their value, it seemed advisable, in connection with the systematic investigations on the chemotherapy of tuberculosis carried on in this laboratory, that the value of mercury and its compounds should be investigated in considerable detail.

In the internal use of compounds of mercury, it is necessary to consider (1) the stability of the preparation in the animal tissues, (2) its method and form of absorption and excretion, (3) the place of deposition of the part of the compounds which is not excreted, and (4) the toxicity and pathogenicity of the preparations.

Morley⁶ states (1) that French workers claim that mercurials are converted in the stomach and intestines into mercuric chloride and circulate as the double salt of mercury and sodium; (2) that it is proved that mercury is absorbed and escapes as an albuminate in every excretion of the body, especially in the urine; (3) that a single dose is excreted within 24 hours; (4) that it accumulates, if given in small doses, and is deposited in all the organs. Blumenthal and Oppenheim⁷ state that after the organic compounds of mercury used by them, a deposit of mercury was found constantly in the liver; sometimes in the intestines and occasionally in the lungs and in the blood. Schamberg, Kolmer and Raiziss⁸ state (1) that the toxicity of mercurial salts is directly proportionate to the amount of mercury contained, the differences of molecular structure being of relatively little importance as affecting their toxicity; (2) that insoluble preparations injected intramuscularly are absorbed at the rate of a little over 1% per day; (3) that after 6 or 7 weeks, almost 50% of the mercury of insoluble preparations may be unabsorbed at the site of injection; and (4) that mercury has a great affinity for the cells of the kidney, and that this organ is one of the earliest involved in mercurial intoxication. All other workers do not agree with Schamberg, Kolmer and Raiziss on the relation of molecular structure to toxicity.

The bactericidal action of mercury has long been recognized, but its use as an internal antiseptic has been limited by its toxicity. Hence the efforts of chemists have for years been turned largely to the construction of mercuric compounds which should be nontoxic or less toxic than mercuric chloride, which kills, on the average, in intravenous doses above 4 mg. per kilogram of body weight, according to Sansum.⁹ Abelin¹⁰ states that the toxic influ-

⁵ Vehr. d. X Internat. Med. Kong., 1890-91, I, Berlin. klin. Wchnschr., 1890, 27, p. 736.

⁶ Calif. State Med. Jour., 1909, 7, p. 338.

⁷ Biochem. Ztschr., 1914, 65, p. 460.

⁸ Boston Med. & Surg. Jour., 1915, 162, p. 826.

⁹ Jour. Am. Med. Assn., 1918, 70, p. 824.

¹⁰ Deutsch. med. Wenschr., 1912, 38, p. 1822.

ence of mercury compounds is in certain relation with their chemical structure, that the toxicity can be diminished by introduction of sulpho or sulph-amino groups or through double carbon connection of mercury, and that easily ionizable mercury compounds are more toxic than the less easily ionizable. Schrauth and Schoeller¹¹ showed that the disinfectant powers of organic mercury compounds were increased by substitution of the less acid phenolic hydroxyl for the carboxyl. They also showed that of the three isomeric mercuriated cresols, the meta derivative is the most potent disinfectant, while the ortho-hydroxy-mercuriphenoxide is more active than the para compound. Also, the entrance of a second hydroxymercuri group increases disinfectant power.

In the work, then, with new mercury compounds it has been necessary to keep the following factors in mind:

1. Minimal toxicity for animal tissues, i. e., organotropism.
2. Maximal disinfectant power
3. Stability or minimal ionization
4. Solubility in nontoxic and nonirritant solvents

This report, which will be followed by others on the same subject, concerns the bacteriostatic and therapeutic effect of some twenty-four inorganic and organic compounds of mercury on the human tubercle bacillus and on experimental tuberculosis in guinea-pigs.

Table 1 gives a list of the compounds reported in this paper, with their chemical formulae, when this was available, and their computed percentage content of mercury.

Part of these compounds have been purchased from Merck and Company, while many have been made for me by the following assistants working in the chemical laboratory of the University of Chicago: Walter Frankel, C. E. Cutler, S. M. Cadwell, Gladys Leavell, L. M. Larsen, Morris Kharasch, Friedrich Lommen, I. M. Jacobsohn.

The inhibitory or bacteriostatic power of most of these mercurials was tested in the usual way by adding the required amount of stock solution of the compound to tubes of melted agar and shaking until well mixed; the tubes were then slanted and cooled and inoculated with human tubercle bacilli, control tubes being made from the same culture. Certain of the compounds could not be dissolved in hot or cold water or in hot agar. Some of these could be dissolved in dilute alkali, in which case the stock solutions were made in either normal sodium hydroxide or 5% sodium carbonate (anhydrous) and diluted with water twenty times. The same amount of the alkali was also added to the control tubes to determine whether the alkalinity used had any inhibitory effect on the growth of the organisms. Some of the compounds, as shown in column 2 of table 2, were insoluble in any known solvent that could be used in this experiment. The required

¹¹ Ztschr. f. Hyg. u. Infektionskr., 1916, 82, p. 279.

TABLE 1
LIST OF COMPOUNDS

Name of Compound	Percentage of Mercury	Chemical Formula
Mercurous Chloride	84.92	$(\text{Hg}-\text{Cl})_2$
Mercuric Chloride	93.78	$\text{Hg}-\begin{array}{c} \text{Cl} \\ \text{Cl} \end{array}$
Mercuric Sulpho-Cyanide	63.29	$\text{Hg}-\begin{array}{c} \text{S}-\text{C}\equiv\text{N} \\ \text{S}-\text{C}\equiv\text{N} \end{array}$
Mercury Potassium Cyanide.....	68.72	$\text{Hg}(\text{CN})_2 \cdot 2 \text{KCN}$
Allyl Alcohol Mercuric Acetate.....	63.3	$\begin{array}{c} \text{H}-\text{C}-\text{Hg}-\text{O}-\text{C}-\text{CH}_3 \\ \quad \quad \\ \text{H}-\text{C} \quad \quad \text{O} \\ \\ \text{H}_2\text{C}-\text{OH} \end{array}$
Mercuric Succinimide	50.5	$\left(\begin{array}{c} \text{H}-\text{C}-\text{C}=\text{O} \\ \quad \quad \\ \text{H}-\text{C}-\text{C}=\text{O} \\ \quad \quad \\ \text{H} \quad \quad \text{N} \end{array} \right)_2 \text{Hg}$
Mercuric Salicylate	42.2	$\begin{array}{c} \text{HO}-\text{C}_6\text{H}_4-\text{C}(=\text{O})-\text{O}-\text{Hg}-\text{O}-\text{C}(=\text{O})-\text{C}_6\text{H}_4-\text{OH} \end{array}$
Mercurio-Iodo Hemol (Merck).....	12.0	Patented Preparation of Hemoglobin, Mercury and Iodin
Mercuriol (Merck)	10.0	Patented Preparation of Mercury and Nucleinic Acid
Mercury Phenolphthalein	39.0	$\begin{array}{c} \text{C}_6\text{H}_5-\text{C}(\text{O})-\text{C}_6\text{H}_4-\text{O}-\text{Hg}-\text{O}-\text{C}_6\text{H}_4-\text{C}(\text{O})-\text{C}_6\text{H}_5 \end{array}$
Mercury Tetraiodphenolphthalein ...	19.6	$\begin{array}{c} \text{C}_6\text{H}_4-\text{C}(\text{O})-\text{C}_6\text{H}_4-\text{O}-\text{Hg}-\text{O}-\text{C}_6\text{H}_4-\text{C}(\text{O})-\text{C}_6\text{H}_4-\text{I} \end{array}$
Fluorescein Mercuric Chloride.....	33.17	$\begin{array}{c} \text{C}_6\text{H}_4-\text{C}(\text{O})-\text{C}_6\text{H}_4-\text{O}-\text{Hg}-\text{O}-\text{C}_6\text{H}_4-\text{C}(\text{O})-\text{C}_6\text{H}_4-\text{OH} \end{array} \cdot \text{HgCl}_2$
Trypan Blue Mercuric Chloride.....	15.65	$\begin{array}{c} \text{NH}_2\text{OH} \\ \\ \text{N}=\text{N}-\text{C}_6\text{H}_4-\text{CH}_3 \\ \\ \text{NaO}_3\text{S} \end{array} \cdot \text{HgCl}_2$
Ortho-Oxy-Benzylidene Amino Phenyl-para Mercuric Acetate.....	44.0	$\begin{array}{c} \text{OH} \quad \text{H} \\ \quad \\ \text{C}_6\text{H}_4-\text{C}=\text{N}-\text{C}_6\text{H}_4-\text{Hg}-\text{O}-\text{C}-\text{CH}_3 \\ \\ \text{O} \end{array}$
1-Amino-2 [para-Naphthalin-Azophenyl Mercuric Acetate] 5 Sulphonic Acid	34.2	$\begin{array}{c} \text{NH}_2 \\ \\ \text{N}=\text{N}-\text{C}_6\text{H}_4-\text{SO}_3\text{H} \\ \\ \text{H}_2\text{O}-\text{C}-\text{CH}_3 \\ \\ \text{O} \end{array}$
Methylene Blue-Mercuric Chloride...	28.94	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{Cl} \end{array} \cdot \text{HgCl}_2$
Iod-Methylene Blue Mercuric Chloride	24.75	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{I} \end{array} \cdot \text{HgCl}_2$
Methylene Green Mercuric Chloride..	31.6	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{NO}_2 \end{array} \cdot \text{HgCl}_2$
Diazo-Amino Methylene Blue ortho-Toluidin di Mercuric Chloride....	29.7	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{Cl} \end{array} \cdot \text{HgCl}_2$
Diazo-Amino Methylene Blue Brom Hydrate ortho-Toluidin Mercuric Bromide	39.5	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{Br} \end{array} \cdot \text{HgBr}$
Diazo-Amino Methylene Blue ortho Phenol Mercuric Chloride.....	29.7	$\begin{array}{c} \text{N}=\text{N} \\ \quad \\ \text{C}_6\text{H}_4-\text{S}-\text{C}_6\text{H}_4-\text{N}(\text{CH}_3)_2 \\ \\ \text{Cl} \end{array} \cdot \text{HgCl}$

amount of such insoluble compounds was weighed into each tube of melted agar. The tubes were well shaken and cooled quickly in ice water so that time was not given for the drug to settle out. In this way a fine suspension of the drug in agar was used, and, as can be seen from table 2, such fine suspensions showed quite as great bacteriostatic power as did the true solutions. It is probable that this is due to a slight unrecognizable solubility, so that in a dilution of 1:20,000 the compounds were really in solution. Only in the case of iodo-mercuro-hemol did even this method fail to give good results. When the hot melted agar was added to the powdered mercuro-iodo-hemol, it formed large, coarse, flocculent masses, which would not break up again. This probably explains the fact that only a dilution of 1:1,000 completely inhibited while a dilution of 1:5,000 partially inhibited growth and that there was good growth on all the higher dilutions. Hence, it required 0.12 mg. of mercury in the form of mercuro-iodo-hemol per c c of agar for complete inhibition of growth, far the largest amount required in any of the tests. Mercuric chloride stood next in the amount of mercury per c c needed for inhibition. This seemed surprising, as mercuric chloride is usually considered high in bacteriostatic power. However, I found it impossible to prepare my agar tubes of mercuric chloride without a certain amount of precipitate in the bottom of the tubes. This consisted of metallic mercury, and, of course, considerably reduced the percentage of mercuric chloride in the agar. However, Schamberg and his co-workers¹ also found that many of their organic mercury compounds had a much higher germicidal power than did mercuric chloride, and in their tests the dilutions were made in distilled water. The calculations of the amount of mercury per c c were made in order to determine whether the inhibitory action on the growth of the tubercle bacillus depends solely on the proportionate amount of pure mercury in the molecule, as Schamberg⁸ found was true of the toxicity, or whether, as Schamberg¹ found in his bactericidal studies with *B. typhosus* and *Staph. aureus*, "the antiseptic value is not related to the amount of pure mercury in the substance, but rather to the chemical constitution of the molecule." By examining the last column of table 2, it may be seen that 4 compounds are, in proportion to the mercury content, considerably higher than the others in bacteriostatic power. These are in order: (1) allyl alcohol mercuric acetate, (2) double salt of methylene blue and mercuric chloride, (3) fluorescein and mercuric chloride and (4) 1-amino, 2-(paranaphthalin azophenyl) mercuric acetate) 5 sulphonic acid. The second and

third are double salts of mercuric chloride and a dye which alone has considerable inhibitory power. This is especially true of methylene blue. In the first, mercury is bound to a carbon of an alcohol ring, while in number 4, mercury is bound by one bond to a carbon of a phenyl ring. The double salt of the dye trypan blue and mercuric chloride requires over twice as much mercury per c.c. to inhibit as the double salt of methylene blue and mercuric chloride, but the dye trypan blue has itself practically no inhibitory power. It seems necessary to conclude from these findings that the different constituents of the molecule play their own rôle in the bacteriostatic action of the mercury compounds. The question of the relation of the position of the mercury in the molecule to bacteriostatic and bactericidal action will be discussed in another paper.

TABLE 2
BACTERIOSTATIC POWER

Mercurial	Soluble in	Growth in Tubes								Mg. Hg per C c Needed to Inhibit
		1: 1000	1: 5000	1: 10,000	1: 20,000	1: 50,000	1: 100,000	1: 500,000	Con- trol	
Mercuric chloride	Water	None	None	None	None	Good	Good	Good	Good	0.093
Mercury sulpho- cyanide	Insoluble, good sus- pension	None	None	None	None	Good	Good	Good	Good	0.031
Mercury potas- sium cyanide	Water	None	None	None	None	Good	Good	Good	Good	0.034
Allyl alcohol mer- curic acetate	Dilute alkali	None	None	None	None	None	Very slight	Slight	Good	0.012
Mercuric succini- mide	Water	None	None	None	None	Good	Good	Good	Good	0.025
Mercuric salicy- late	Insoluble, good sus- pension	None	None	None	None	Good	Good	Good	Good	0.021
Mercurio-iodo- hemol	Insoluble, poor sus- pension	None	Slight	Good	Good	Good	Good	Good	Good	0.12
Mercuriol (mercu- ric nucleinate)	Water	None	None	Slight	Good	Good	Good	Good	Good	0.02
Mercury phenol- phthalein	Dilute alkali	None	None	None	Good	Good	Good	Good	Good	0.039
Fluorescein mer- curic chloride	Dilute alkali	None	None	None	None	Good	Good	Good	Good	0.016
Ortho-oxyben- zylidene amino phenyl para mercuric acetate	Dilute alkali	None	None	None	None	Good	Good	Good	Good	0.022
1-amino-2(p-naph- thalin azo- phenyl mercuric acetate) - 5 sul- phonic acid	Dilute alkali	None	None	None	None	Slight	Slight	Very slight	Good	0.017
Trypan blue mer- curic chloride	Slightly in hot water	None	None	Good	Good	Good	Good	Good	Good	0.03
Methylene blue mercuric chloride	Insoluble, good sus- pension	None	None	None	None	Slight	Good	Good	Good	0.014
1:0 methylene blue mercuric chloride	Insoluble, good sus- pension	None	None	None	Very slight	Good	Good	Good	Good	0.024

It will be seen that all the compounds showed some inhibitory power, that nearly all inhibited completely at 1:10,000, while many also inhibited at 1:20,000.

With only 3 compounds, allyl alcohol mercuric acetate, double salt of methylene blue and mercuric chloride, and 1-amino, 2 (p-naphthalin azo-phenyl mercuric acetate) 5 sulphonic acid, did the bacteriostatic action go farther than 1:20,000.

The bactericidal power of the compounds used in these experiments has not been tested, partly because of the insolubility or difficult solubility of most of the preparations. In most of the experiments with antiseptics and drugs used in my former experiments, we have found that a somewhat lower concentration was required for inhibition than for complete bactericidal action. In a report by DeWitt and Sherman,¹² we found that human tubercle bacilli distributed in a thin layer on garnets and exposed for 24 hours to mercuric chloride in a dilution of 1 part of the drug to 100,000 parts of water were killed so that they failed to infect guinea-pigs.

It required 1 part in 50,000 to kill them in clumps in 1 hour so that they would not grow on agar tubes and 1 part in 1,000 of water to kill them in 1 hour so that they would not cause the disease in animals. In all cases, the garnets, after exposure to the mercuric chloride solutions, were well washed with ammonium sulphide solution to neutralize the mercury and then with 4 wash waters, so that none or very little of the drug could have been carried over to continue its action. It is difficult to explain this apparently much higher bactericidal than bacteriostatic power of mercuric chloride except on the assumption that a considerable part of the mercury must have been thrown out of solution in the agar. However, Lewis¹³ concludes that there is no close or constant correlation between the bactericidal and inhibitory action of chemicals.

THERAPEUTIC EXPERIMENTS

1. Mercurous Chloride

Six guinea-pigs were inoculated subcutaneously with 0.2 c.c. of a suspension of human tubercle bacilli of the strain known as "Old Human" to distinguish it from the various strains acquired more recently. The suspension was diluted to the point at which it was just slightly opalescent. These 6 animals were each fed daily one pill containing 1 mg. of mercurous chloride. One pig died in 2 days, before any tuberculous involvement was possible. The others lived

¹² J. Infect. Dis., 1914, 15, p. 245.

¹³ J. Exper. Med., 1917, 25, p. 441.

167, 231, 240, 245, and 325 days, an average of 241 days, and all showed marked tuberculous involvement of lymph glands, spleen, liver and lungs. They had received by mouth 125, 198, 209, 213, and 282 mg., an average of 205 mg. Control pigs were inoculated on the same day with the same dose of the same suspension and lived 85, 183, 193, 193, and 193 days, an average of 169 days. All the control animals also showed marked generalized tuberculosis. The only difference, then, between the treated and the untreated animals was that the treated animals lived longer than the untreated, but the extent of the disease at death was about the same.

2. *Mercuric Sulphocyanide*

Six guinea-pigs were inoculated with 0.2 c.c dilute suspension of the strain known as "Old Human." They received one subcutaneous injection of 0.5 mg. and one of 1 mg. and were fed 1 mg. of the drug per day. The compound was toxic enough so that daily feedings had to be interrupted at intervals for a week or more and then resumed. Two of the animals received one intracardiac injection of 1 mg. One of these died the next day and the other 33 days later. The length of life after inoculation was 42, 85, 96, 143 and 193 days. The average length of life was 112 days. The amount of the drug administered was 39.5, 60, 93, 124, 166 mg., or an average per animal of 91.5 mg. All the animals had enlarged caseous inguinal glands. The one dying in 42 days had no other tubercles visible macroscopically. The others showed some tubercles in the organs but only in the one living 193 days was the disease marked.

The duration of life of the untreated controls was 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days. Hence with this treatment, the treated animals died more quickly than the untreated controls. On the other hand, the extent of the disease in all the controls was much greater than in the treated animals, the liver, spleen and lungs in those dying after 98, 111, 116, and 117 days being more involved than in the treated animals living 143, and 193 days. This suggests that the drug had a certain slight inhibitory action over the progress of the disease although not enough to prevent it completely, while its toxic action was sufficient to shorten life in many of the animals.

3. *Mercury Potassium Cyanide*

Six guinea-pigs were inoculated with 0.2 c.c of dilute emulsion of "Old Human" tubercle bacilli. Each received one subcutaneous, one intracardiac and one intramuscular injection and was fed 1 mg. per day. One died on the eighth day after inoculation with no sign of tuberculosis. One died on the thirty-second day showing an enlarged regional gland and a few small tubercles in the spleen. The others lived 86, 162, 169 and 238 days, or an average of 166 days. All showed marked tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days, and all had a marked generalized tuberculosis. In this series, if we leave out of consideration 2 treated animals that died early from some cause other than tuberculosis, the treated animals lived longer than the controls, but the extent of the disease was about the same in the two sets.

4. *Allyl Alcohol Mercuric Acetate*

This preparation was made for me in the chemical laboratory of the University of Chicago. My reason for having it made was the reputation that allyl alcohol and some of its derivatives have acquired in the clinical treatment of tuberculosis. As is seen in table 2, this mercurial compound of allyl alcohol has a high bacteriostatic power over the growth of the human tubercle bacillus. The compound could be dissolved in water to make a solution of one part in 1,000. Six guinea-pigs were inoculated with 0.05 mg. of culture strain 1305, isolated by Dr. H. J. Corper at the Chicago Municipal Sanatorium about 5 years ago. Each animal received a subcutaneous injection of 1 mg. of the allyl alcohol mercuric acetate in 1 c.c. of water. Then each was fed 1 mg. pill per day up to the time of death. These animals lived 46, 56, 99, 102, 104 and 140 days, or an average of 91 days. They had received 41, 49, 87, 87, 88 and 118 mg. of the drug, or an average of 78 mg. for each animal. All showed marked tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 77, 78, 79, 84, 119 and 119 days, or an average of 92.6 days. All the animals showed marked generalized tuberculosis. In spite then, of the high bacteriostatic power of allyl alcohol mercuric acetate in vitro, it seems to have no therapeutic effect on animals inoculated with a virulent strain of tubercle bacilli, since both length of life and extent of disease are practically the same in the treated and untreated animals. It is probable that this drug fails to penetrate the young tubercles, or is broken up and loses its antiseptic power before it reaches them.

5. *Mercuric Succinimide*

This is the mercury compound which, perhaps because of its being a soluble organic mercury compound, has been especially used in the clinical treatment of tuberculosis. It was used largely by B. L. Wright and his followers, although some of them substituted mercuric chloride for the succinimide. Six guinea-pigs were inoculated with 0.2 c.c. of the dilute suspension of the "Old Human" strain of tubercle bacilli. Subcutaneous and intracardiac injections were used at first, then pills containing 1 mg. of the drug were fed daily. One lived only 10 days, succumbing on the day following an intracardiac injection and, of course, showed no tuberculosis. The others lived 85, 95, 154, 189 and 219 days, an average of 148 days. They received 84, 90, 154, 214 and 240 mg. of the drug, or an average of 156 mg. The extent of the disease was distinctly limited in this series. The pig that died in 85 days showed only an involvement of the regional lymph glands with necrotic masses in the spleen. Even the one that lived the longest showed but slight involvement of the spleen and lungs and regional lymph glands. Some had partial necrosis of the liver and some showed cirrhosis of the liver, but the involvement of the organs was slight for the duration of the disease. The controls lived 98, 111, 116, 117, 143 and 156 days, or an average of 120 days. All had marked tuberculosis of lymph glands, spleen, liver and lungs. The treated animals have lived on the average somewhat longer than the controls, although 2 died earlier and 2 lived much longer than any of the controls. The extent of the disease in the treated was considerably less than in the untreated animals.

6. *Mercuric Salicylate*

Five guinea-pigs were inoculated with 0.2 cc of dilute suspension of Miller's strain of human tubercle bacilli, a moderately virulent strain. Since mercuric salicylate is insoluble, a suspension was made in sterile paraffin oil and an amount containing 2 or 3 mg. of the drug injected intramuscularly once a week. On the other days of the week, a pill, containing at first 1 mg. and afterward 2 mg., was fed to each animal. This dosage was evidently too toxic, as the animals lived only 13, 27, 27, 37 and 59 days, having received 12, 24, 24, 35 and 67 mg. of the drug. The inguinal glands were enlarged and caseous in all except the one dying in 13 days, and the spleen was involved in some of the others. Hence, even in these doses which so quickly killed the animals, there was evident little, if any, influence on the progress of the infection. The controls of this series lived 91, 96, 97, 104, 121 and 226 days, an average of 122.5 days, and all showed marked generalized tuberculosis.

7. *Mercurio-Iodo-Hemol*

This is an insoluble commercial preparation obtained from Merck and Company. Hemol is a hemoglobin preparation, and the compound used is said to contain 12.35% of mercury and 28.6% of iodine. From 2-5 grains by mouth three times per day is the dose recommended for syphilitic patients. Six guinea-pigs were inoculated with 0.2 cc of a dilute suspension of the "Old Human" strain of tubercle bacilli. Each animal was fed daily a pill containing 1 mg. of the mercurio-iodo-hemol preparation and received several subcutaneous injections of a suspension in gelatin and an intramuscular injection. They lived 67, 96, 110, 133, 135 and 171 days, or an average of 120 days, and received 41, 86, 97, 109, 119 and 147 mg. of the drug, an average of 99% mg. All showed considerable tuberculous involvement of the lymph glands, liver, spleen and lungs. The controls of this series lived 98, 111, 116, 117, 143 and 156 days, or an average of 123.5 days, and all showed an extreme degree of generalized tuberculosis. Hence, the hemol mercury preparation, while not especially toxic, seems to have had no beneficial influence on the progress of the disease.

8. *Mercuriol*

Mercuriol or mercury nucleide is also a commercial preparation obtained from Merck and Company. It is soluble in water and is said to contain 10% of mercury. It was tested in this series of experiments, especially because of the favorable reputation which nuclein and nucleinic acid have long borne in the clinical treatment of pulmonary tuberculosis. The mercuriol is freely soluble and neither very toxic nor locally irritating, so that injections of various kinds could be continued much longer than with most of the mercurial preparations. Several sets of animals were therefore tried out in testing the therapeutic properties of this drug.

(a) Six guinea-pigs were inoculated with 0.2 cc of a dilute suspension of "Old Human" tubercle bacilli. These were fed daily pills containing each 1 mg. of the drug, the daily dosage being increased according to the condition of the animal. They also received injections once a week, 2 subcutaneous injections of 1 and 3 mg. and 4 intracardiac injections of 2, 3, 4 and 5 mg. of the drug. They lived 33, 42, 47, 56, 82 and 234 days, or an average of 82 days. They received 36, 49, 57, 76, 129 and 349 mg., or an average of 116 mg. of the drug. Three of these animals died as a direct result of an intracardiac injection, one died from a septic abortion, while none had

any considerable tuberculous involvement more than enlarged and partly caseous regional lymph glands. The controls of this set lived 26, 56, 71, 137, 202 and 217 days, or an average of 118 days, and all except the first two to die had marked generalized tuberculosis.

(b) The second set consisted of 6 guinea-pigs inoculated with the same dose of the same strain as set 1. They were treated merely by feeding mercuriol pills, gradually increasing the dose as the condition of the animal permitted. Two other guinea-pigs were inoculated with the caseous substance from a lymph gland taken from a pig of set 1 and were treated with the pigs of set 2, after a local tubercle developed. These lived 48, 90, 122, 139, 160, 271, 271, 271 days, or an average of 171 days. They received by mouth 42, 86, 166, 199, 186, 186, and 186 mg. In all these animals there were found enlarged caseous glands in the inguinal region and from slight to moderate involvement of the liver, spleen and lungs. In none of the animals of this set were these organs normal or even completely free from tubercles, as they were in set 1. The controls of this set lived 85, 183, 193, 193 and 193 days, or an average of 169 days, a slightly lower average length of life than in the treated animals because 3 of the treated animals lived much longer than the others and much longer than any of the controls. All of the controls, except the one dying on the eighty-fifth day, exhibited much more marked general tuberculosis than did the treated animals.

(c) The third set was inoculated with the same dose of the same strain as the other two sets. It was treated by feeding and intraperitoneal injections. These died in 47, 78, 118, 159 and 164 days, or an average of 113 days. They had received 52, 78, 126, 142 and 155 mg., or an average of 111 mg. of the drug. The one dying after 47 days had enlarged caseous glands in both groins and a few in the spleen and liver. The one dying in 78 days had no caseous glands and no macroscopic tubercles in the liver, spleen or lungs. The 3d, 4th and 5th to die showed no tubercles in any of the organs and no caseous glands. This pathologic condition would have seemed somewhat encouraging for the claims of mercuriol had it not been that the control animals of that set lived much longer than the treated and exhibited about the same degree of tuberculous involvement. This seemed to indicate that the "Old Human" strain was losing its virulence to a degree which made it hardly usable for therapeutic experiments. In connection with set 3, a number of uninoculated guinea-pigs were treated with mercuriol in the same way as the infected pigs. These pigs were killed after the tuberculous pigs had died. No pathologic changes were noted, except hemorrhages and hemorrhagic exudates in most of the animals.

In regard to mercuriol, we must conclude that, while it is quite nontoxic and nonirritating and seems to have some slight inhibiting influence over the spread of the disease in the animal, that influence is not sufficient to encourage us in further experiments, since in all probability, with more virulent strains, the influence of the drug would either not be felt or, as often happens, would stimulate the organism to increased growth and virulence.

9. Mercury Phenolphthalein

This preparation was made for me in the chemical laboratory of the University of Chicago according to suggestions made by Hahn and Kostenbader.¹⁴ It is insoluble in water but dissolves in alkalis. The mercury makes up about 39% of the molecular weight. (See table 1.)

¹⁴ Ztschr. f. Chemotherapie, O., 2, O, p. 71.

Six guinea-pigs were inoculated with 0.2 cc of a dilute suspension of "Old Human" strain of tubercle bacilli and each was fed daily first one and later two pills containing each 2.6 mg. Three of the 6 animals died during the first month of some cage infection with no sign of tuberculosis. The other three lived 210, 244 and 306 days and, at death, showed advanced tuberculous involvement of lymph glands, liver, spleen and lungs. The controls died after 126, 170, 182, 187, 192 and 205 days, all showing marked general tuberculosis.

10. *Mercury Tetraiod Phenolphthalein*

This was also suggested by Hahn and Kostenbader and, having 4 iodine atoms in the molecule given for mercury phenolphthalein, was also tested, the animals being inoculated and then fed daily. Aside from 2 animals that died early, they lived 133, 151, 166 and 241 days, and all showed at death advanced tuberculosis of lymph glands, spleen, liver and lungs. The controls lived 122, 133, 150, 169, 176 and 295 days. All had extensive advanced tuberculosis. Hence these 2 phenolphthalein dyes seem to have no influence on the extent and distribution of the disease.

11. *Double Salt of Fluorescein and Mercuric Chloride*

This also is one of Hahn and Kostenbader's "mercurialized dyes." Six guinea-pigs were inoculated with 0.2 cc of a dilute suspension of the "Old Human" strain and then fed with the dye. Three died during the first month of cage infections. The other three lived 58, 88 and 217 days having received 250, 426 and 950 mg. of the dye. The one living 58 days showed no generalized tuberculosis, the second contained tubercles only in the lungs, while the third had only a few small tubercles in the lungs, but the liver and spleen were full of tubercles. The controls lived 126, 170, 182, 187, 192 and 205 days, or an average of 177 days, and all showed extensive advanced general tuberculosis.

In spite of the fact that the involvement in the controls was much greater than in the treated pigs, we cannot under these conditions consider that the difference was due to the treatment, since all animals that lived as long as did the controls had some generalized tuberculosis, even though not distributed through all the organs.

12. *Double Salts of Trypan Blue and Mercurous Chloride and Mercuric Chloride*

These salts were made for me by Dr. Walter Fraenkel, and the method of making was described in a previous paper.¹⁵ At that time it was found that these salts had some tuberculocidal power since 0.1 cc of a suspension of tubercle bacilli exposed for 24 hours to 1% solution of the salts and then injected into guinea-pigs failed in most cases to cause disease. The inhibiting action of the mercuric salt was surprisingly low, since only the 1:1,000 and 1:5,000 dilutions caused complete inhibition. Six guinea-pigs were inoculated with 0.2 cc of dilute suspension of the "Old Human" strain. They received the mercurous salt of the dye both by daily feeding of 1, 2 or 3 mg. and by occasional intramuscular injections of a suspension in oil. They lived 33, 35, 38, 73, 93 and 219 days, or an average of 81 days. They had received

¹⁵ Jour. Infect. Dis., 1914, 14, p. 498.

37, 43, 52, 97, 181 and 192 mg., or an average of 100 mg. All showed generalized tuberculosis in moderate degree. A second set was inoculated with 0.1 c.c. of the Miller's, a more virulent strain. These were both fed and injected. They lived 91, 94, 137 and 139 days, or an average of 115 days. They received 110, 112, 137 and 139 mg. of the dye. All showed an advanced generalized tuberculosis. Hence, these mercury salts of trypan blue have only slight bactericidal and inhibitory power and no therapeutic influence over the disease.

13. Mercury Derivatives of Aromatic Amines

Jacobs and Heidelberger¹⁶ described some mercury derivatives of aromatic amines. As some of these preparations looked as if they might be of interest in my work, I had my chemist make two of them according to their directions.

(a) 4-ortho-oxybenzylidene amino phenyl para mercuric acetate, which contains 44.02% of mercury. This compound is soluble in dilute alkali. Six guinea-pigs were inoculated with 0.2 c.c. of a dilute suspension of "Old Human" strain of tubercle bacilli. One mg. pills were fed to each pig daily and several injections were given. Two of the pigs died shortly after an intracardiac injection. The other animals lived 33, 68, 78 and 220 days, or an average of 99 $\frac{3}{4}$ days. They had received 32, 40, 53 and 151 mg., or an average of 69 mg. per animal. The three that died earliest had no or slight tuberculosis, but the one living 220 days showed an extensive generalized tuberculosis. The controls lived 24, 119, 184, 238, 264 and 315 days, or an average of 207 days, much longer than the treated animals and all except the first showed marked and extensive generalized tuberculosis.

(b) 1-amino-2(para-naphthalin azophenyl mercuric acetate) 5-sulphonic acid. This contained 34.24% of mercury and was also soluble in dilute alkali. The inhibitory power was high, since there was complete inhibition up to 1:20,000 and partial inhibition even to 1:500,000. Two sets of animals were used for this compound. The first set was inoculated and treated in the same way as the set just described. These lived 10, 30, 50, 51, 65 and 250 days. The first ones died from the effect of an injection. Only the one that died last after 250 days showed tuberculosis. The second set was inoculated with the same dose of the same strain and treated mostly by feeding, only one subcutaneous injection having been given. These animals lived 36, 118, 146, 155, 188 and 255 days, an average of 149 days. All showed slight to moderate generalized tuberculosis with considerable tendency to the fibrous form of tuberculosis. The controls lived 42, 168, 199, 219, 276 and 281 days, or an average of 197 days, much longer than the treated pigs, and all except the first showed an advanced generalized tuberculosis.

14. Mercury Compounds of Methylene Blue and Its Derivatives

This group of compounds has been of especial interest to me because of the position methylene blue itself has occupied as an internal germicide and also in the treatment of tuberculosis. Some of the work has already been reported briefly.¹⁷ In this present report, the work of the various mercuric compounds of the group of dyes related to and derived from methylene blue will be reported more in detail. In the earlier work and by different methods than those

¹⁶ J. Biol. Chem., 1914, 20, p. 513.

¹⁷ DeWitt: Jour. Infect. Dis., 1913, 13, p. 178; Transactions of the Twelfth Annual Meeting of the National Tuberculosis Association, 1916.

used, I found the methylene blue chloride caused complete inhibition of the human tubercle bacillus in a dilution of 1:5,000, while Paul Lewis¹⁸ by still another method found that 1:1,000,000 was the highest concentration at which the growth equaled that on control flasks. Methylene blue was also shown by me to have some bactericidal action on the tubercle bacillus.

(a) The double salt of methylene blue and mercuric chloride, which was made by my chemical assistants by adding a solution of mercuric chloride to a solution of methylene blue and thoroughly washing and drying the precipitate thus formed, contains about 34% mercury and is quite insoluble, but forms a good suspension on shaking in hot agar and then cooling and slanting quickly. There was no growth in tubes up to 1:20,000 and only slight growth in the tubes containing a dilution of 1:50,000. The amount of mercury per cc in the highest dilution which completely inhibited was 0.014, which was less than completely inhibited in any other mercurial tested, except the allyl alcohol mercuric acetate. In the first experiment with this drug, 4 of the 6 guinea-pigs died within the first 3 weeks. The other 2 lived 130 and 208 days. Each pig was fed 1-2 mg. every day during life and received injections once a week. There was practically no tuberculous involvement even in the animal that lived 208 days. Another set of animals was therefore inoculated in the same way with 0.2 cc of dilute suspension of the "Old Human" strain of tubercle bacilli. These received no injections but were fed daily pills containing a dose of 1-2 mg. These pigs lived 147, 237, 265, 269 and 288 days, an average of 241 days. They received by mouth during life 264.6, 460, 460, 500 and 520 mg. of the drug, or an average of 440.9 mg. per animal. Four of these pigs showed at necropsy only slight involvement of regional lymph glands and of the spleen; but the fifth showed extensive and advanced tuberculosis of lymph glands, spleen, liver and lungs. The controls of this series lived 126, 170, 182, 187, 192 and 205 days, an average of 177 days, a much shorter time than the treated animals lived, while every one of the controls showed marked, extensive and advanced tuberculosis of lymph glands, spleen, liver and lungs. These results were so good that it seemed well worth while to carry the experiment further, and another set of guinea-pigs was inoculated with the same dose of the same strain of human tubercle bacilli. These also were fed daily and no injections were given. These lived 96, 188, 247, 267, 298 and 298 days, an average of 231 days. In 4 of these the tuberculous involvement was slight, while in 2 it was extensive. The controls of this series lived 42, 168, 199, 219, 276 and 281 days, an average of 197 days, and all except the first showed as extensive tuberculosis as the worst two of the treated animals. The effect of treatment in this set seemed much less than in the other sets described, but the treated animals lived longer and showed less extensive disease than the untreated.

The next set consisted of 5 guinea-pigs inoculated with 0.2 cc dilute suspension of the "Old Human" strain. They received the drug by daily feeding of 1-2 mg. pills and intramuscular injection of the drug in cotton seed oil. They lived 100, 147, 265, 335 and 733 days. None of these animals had any definite tubercles in any of the internal organs, though all had slightly enlarged noncaseous glands in the inguinal region. The controls of this set lived 145, 172, 222, 232 and 266 days, an average of 207 days, and all had advanced tuberculosis of lymph glands, liver, spleen and lungs. The next set consisted

¹⁸ Jour. Exper. Med., 1917, 25, p. 441.

of 6 guinea-pigs inoculated with the same dose of the same strain as the other sets and treated by daily feedings and weekly intramuscular injections of the double salt in cotton seed oil. They lived 97, 101, 103, 161, 402, 813 and 1,000 days. The 2 that lived over 2 years showed no sign of tuberculosis except slightly enlarged and hard glands in the groins, which showed necrotic fibrous encapsulated tubercles. The others showed no sign of tuberculosis, except some enlarged, noncaseous glands. This would have been satisfactory had it not been that the controls of this set, although they died much earlier than some of the treated animals, living 110, 160, 204, 233 and 234 days, and although they had enlarged and caseous glands, showed but slight or moderate tuberculosis of the internal organs. It seemed safe to conclude from all these experiments that animals inoculated with a strain of low virulence certainly received considerable benefit from treatment with small doses of the double salt of methylene blue and mercuric chloride. A much more virulent strain was then used to determine whether the effect observed in the animals inoculated with the strain of low virulence would also be observed in those inoculated with a strain of high virulence. The strain used was called "1305" and was one isolated in about 1916 by Dr. Corper from sputum obtained from a patient in the Chicago Municipal Tuberculosis Sanatorium. Eight guinea-pigs were inoculated subcutaneously, each receiving 0.05 mg. of the culture. They were treated by daily feeding of pills containing each 1 mg. of the double salt of methylene blue and mercuric chloride. They lived 54, 58, 70, 76, 77, 111, 121 and 122 days, or an average of 73 days. They had received 42, 50, 60, 65, 66, 98, 104 and 105 mg. of the drug, or an average of 74 mg. per animal. All the animals showed some tuberculosis of the internal organs at necropsy and in nearly all the disease was moderately or extremely extensive. The controls lived 60, 109, 111, 129 and 172 days, or an average of 116 days, and all showed marked tuberculosis of the lymph glands, spleen, liver and lungs. In this set there was practically no difference between the treated and untreated guinea-pigs. Another set was inoculated with 0.05 mg. of 1305, then fed 1 mg. of the double salt daily, as in the last set, but these also received several subcutaneous injections of 1 mg. of egg albumin. These lived 92, 104, 106, 121, 124 and 214 days, and nearly all showed extensive involvement of lymph glands, liver, spleen and lungs. Still another set, inoculated in the same way with the same dose of the same strain as the set described above and as the controls given under the first of these three 1305 sets, received in addition to the feeding with the double salt of methylene blue and mercuric chloride several subcutaneous injections of a vaccine made by heating a suspension of the same strain with which they were inoculated to 60 C. for one hour. These lived 30, 67, 85, 111, 130, 143, 143, 153 and 156 days, or an average of 113 days as compared with an average of 116 days life of the controls. All of the guinea-pigs of this set also showed extensive tuberculosis of the internal organs in a degree that is apparently about equal to that of the control animals.

Another set was inoculated each with 0.05 mg. of the virulent strain 1305 and then fed daily from 1 to 2 mg. of the double salt. They lived 62, 73, 95, 96, 101, 129, 164 and 259 days. They had received by mouth before death 63, 73, 92, 93, 97, 121, 151 and 389 mg. Five of these 8 guinea-pigs showed either no or slight tuberculous involvement, having died from some acute infection. The 2 that died after 96, 101 and 259 days showed a rather extensive general involvement. Six of the 8 controls of this set have died after

14, 66, 66, 207, 213 and 267 days. The one dying after 14 days showed no tuberculosis, and one of those dying after 66 days had a slight degree of the disease; in the other 4 the glands, liver, spleen and lungs were extensively involved.

Hence, we must say that, as far as we may conclude from these experiments, the double salt of methylene blue and mercuric chloride, if fed in 1 mg. doses, exerts no or but little beneficial influence on the rapidly progressive type of tuberculosis caused in guinea-pigs by inoculation with a virulent human strain, although in the type caused by inoculation with a strain of low virulence, it seems to exert a distinct and considerable influence, although it cannot be said to cure, even in these cases. If we may judge by the length of life and by the condition of the animals, we cannot consider that it had in any case an injurious influence if given by mouth and, if given by intramuscular injection, there seems to have been no other ill effect than the local infiltrations and occasional ulcerations usually caused by any oil emulsion.

In addition to the experiments described on the use of the double salt of methylene blue and mercuric chloride, 2 sets of animals were treated with alternate injections of methylene blue and of calomel and one set using alternate injections of methylene blue and of mercuric chloride. This was done with the idea that a compound of methylene blue and mercury might thus be formed internally and perhaps be more efficacious. The first set treated with methylene blue and calomel died during the first month, showing no sign of tuberculosis. The second set received the methylene blue by intracardiac injection and the mercurous chloride by intramuscular injection, but both in smaller individual doses than were used in the first set of animals. Three of the animals of this set died after the first intracardiac injection, 2 from abortion and the others from the heart injection. The other 3 received the injections about once a week for one month and afterward no further treatment and lived 172, 211 and 312 days. Only the last one of these showed any considerable tuberculous involvement. The controls of this set lived 26, 56, 71, 137, 202 and 217 days. The strain used for inoculation in this set was the "Old Human" which had by this time partly lost its virulence, so that only the controls that had lived 200 or more days showed extensive tuberculosis, while in the treated animals, only the one that lived 312 days showed any considerable disease.

The next set were inoculated subcutaneously with 0.2 c.c. of "Old Human" strain of tubercle bacilli and for a little over one month received weekly intraperitoneal injections of methylene blue and mercuric chloride separately. These drugs were also fed to them on alternate days. They lived 41, 65, 106, 106, 110 and 247 days, or an average of 113 days. Those that lived 106 and 110 days showed moderate generalized tuberculosis, but the others, even the one living 247 days, showed none. The controls of this set lived 110, 160, 204, and 233 and 234 days and showed little generalized tuberculosis. All showed enlarged and partly caseous glands. Hence this treatment of methylene blue and mercuric chloride given separately cannot be said to have had any beneficial effect even in the animals inoculated with a strain of human tubercle bacilli of extremely low virulence.

(b) Double salt of iod methylene and mercuric chloride. Iod methylene blue was made by my chemical assistant and was found to inhibit growth of tubercle bacilli in dilutions of 1:5,000, just as did methylene blue itself. It

is probably the same compound recommended by von Linden. The double salt was made by adding a solution of mercuric chloride to a solution of iod-methylene blue and washing the precipitate thus formed. It contains 24¼% of mercury and its inhibiting power was a little less than that of the double salt of methylene blue and mercury, since there was slight growth in the tubes containing the dye in a dilution of 1:20,000, about corresponding to the growth in the tubes containing dilutions of 1:50,000 of methylene blue and mercuric chloride. Six guinea-pigs were inoculated with 0.2 cc of a dilute suspension of "Old Human" strain of tubercle bacilli and then fed the dye daily in 1 mg. doses and injected once a week subcutaneously during the first month. They lived 81, 142, 143, 165, 204 and 453 days, an average of 198 days, and had received 89, 156, 157, 178, 224 and 509 mg. of the dye, or an average of 219 mg. per animal. All the animals of this set exhibited extensive tuberculosis. The controls lived 122, 147, 176, 197, 215 and 238 days, or an average of 182.5 days. The average duration of life of the controls was less than that of the treated animals, but that was due to the one animal of the treated set which lived more than twice as long as any of the others and almost twice as long as the last of the controls to die. The extent of the disease in the controls was about the same as in the treated animals, so that the double salt of iod-methylene blue and mercuric chloride seems to have had in this experiment no beneficial action, although the strain with which the animals were inoculated was one of fairly low virulence. This may partly confirm a statement made by Lenard¹⁹ that iodine prevents the deposition of the mercury in the liver, which is said by Blumenthal and Oppenheim²⁰ to have something to do with the therapeutic power.

(c) Double salt of methylene green and mercuric chloride. Methylene green has one nitro group in the methylene blue molecule. The dye itself has not in my experiments shown itself equal to the methylene blue in inhibitory, bactericidal or therapeutic power. The mercury preparation has been made by my assistants by the same method as the other double salts. It is quite insoluble. Six guinea-pigs inoculated with 0.2 cc of a dilute suspension of "Old Human" strain were fed daily and received several injections. Four of the 6 died within the first month, the other 2 living 201 and 238 days and showing extensive and advanced tuberculosis. Therefore another set was inoculated in the same way and received no injections, but simply daily feedings. These lived 73, 199, 220, 226 and 378 days, an average of 219 days. They had received 114, 398, 413, 450 and 648 mg., or an average of 405 mg. of the double salt. The first of these animals that died showed no tuberculosis except a small gland and a few tubercles in the spleen. The third also showed but a slight degree of tuberculosis. The second and fourth had a moderately advanced tuberculosis and the last exhibited signs of healed and healing tubercles, as the groins contained small calcified granules and in the lungs and liver the tubercles were hard and fibrous. The controls of this set lived 126, 170, 182, 187, 192 and 205 days, or an average of 177 days. All the controls showed extensive, advanced tuberculosis. If we consider in this experiment the consistently longer life of the treated animals, the less extent of the disease, and the tendency to healing shown in the guinea-pig that lived longest, we may conclude that the double salt of methylene green and mer-

¹⁹ Ztschr. f. Chemotherapie, 1914 2, p. 106.

²⁰ Biochem. Ztschr., 1914, 65, p. 460.

curic chloride, as well as that of methylene blue and mercuric chloride, shows some beneficial influence on tuberculosis caused by a strain of tubercle bacilli of relatively low virulence. The strain of high virulence was not tested with this compound.

(d) Diazo amino methylene blue ortho toluidin dimercuric acetate was made for me by my chemical assistant, Doctor Kharasch. The method of making this compound and the two following ones will be reported by him later. The chemical formula for it is given in table 1; it contains 41.3% of mercury, and is quite insoluble. Its inhibitory and germicidal power have not yet been determined. Seven guinea-pigs were inoculated subcutaneously, each with 0.05 mg. of the virulent strain 1305. They were fed daily and received one subcutaneous injection. They lived 8, 32, 100, 106, 137, 140 and 148 days, an average of 96 days, and received 7, 29, 87, 92, 119, 121 and 128 mg. of the compound. All except the 2 that died after 8 and 32 days showed a high degree of advanced generalized tuberculosis. The controls of this set lived 51, 83, 83, 94, 127 and 143 days, or an average of 97 days, and all showed marked and extensive tuberculosis of lymph glands, spleen, liver and lungs. This experiment, therefore, would not indicate that this compound of methylene blue has any beneficial action on this virulent tuberculous infection.

(e) Diazo amino methylene blue brom hydrate ortho toluidene di mercuric acetate. The formula for this is given in table 1, and it contains 39.5% of mercury. It is insoluble. Nine guinea-pigs were inoculated subcutaneously with 0.05 mg. of strain 1305 and then given one injection of the compound suspended in oil and a daily feeding of 1 mg. They lived 8, 23, 32, 76, 81, 95, 129, 133 and 170 days, or an average of 92 days, if we omit the accidental death at 8 days. They had received 10, 21, 64, 69, 81, 131, 125 and 145 mg., or an average of 78 mg. per animal. All except the 3 animals dying after 8, 23, and 32 days, had a pronounced general tuberculosis. Hence this compound, like the same compound without the brom hydrate, has no beneficial influence in this virulent infection.

(f) Diazo amino methylene blue ortho phenol mercuric chloride contains 29.7% of mercury. Ten guinea-pigs were inoculated with 0.05 mg. of strain 1305 and were then fed daily 1 mg. and received one injection of the compound suspended in oil. These lived 11, 16, 18, 48, 72, 126, 144, 154, 171 and 174 days or an average of 127 days, after omitting the first two. They received 14, 19, 21, 35, 55, 101, 117, 125, 126 and 129 mg. of the compound, an average of 74.2 mg. The first 2 died from septic abortion and the third from acute pneumonia. Four of the others showed generalized tuberculosis of moderate degree, while the other 3, which were the last to die, exhibited a slight degree of tuberculosis, a few small tubercles in one of the organs or perhaps only an enlarged gland. The controls of e and f lived 62, 125, 125, 128 and 138 days, or an average of 115 days, and all showed advanced generalized tuberculosis.

Concerning the 3 diazo compounds of methylene blue and mercury just described, but little can be said. A number of the treated animals died early, suggesting a toxic and weakening action of the drug. With the 2 o-toluidin compounds d and e, there was no difference between the treated and untreated animals either in length of life or in extent of the disease. The animals treated with the o-phenol compound f lived slightly longer and had less tuberculous involvement than the controls, although they were inoculated with the virulent strain 1305.

The results of the chemotherapeutic experiments with these 24 mercuric compounds have been described as briefly as possible in the foregoing pages. But, in order to compare these results, a brief summary is given in table 3. It will be seen from this table that, as compared with the untreated controls that had been inoculated with the same dose of the same strain of tubercle bacilli, several showed a greater duration of life and less extent of disease. In nearly all of these, the infection was due to a strain of lower virulence, and when the same drug was tested with a strain of high virulence no effect was noticed.

TABLE 3
SUMMARY OF RESULTS

Mercury Compounds	Strain of Tubercle Bacilli	Length of Life Compared With Controls	Extent of Disease Compared With Controls
Mercurous chloride	O. H.	Greater	Same
Mercury sulpho cyanide.....	O. H.	Slightly less	Less
Mercury potassium cyanide.....	O. H.	Sl. greater	Same
Allyl alcohol mercuric acetate.....	1305	Same	Same
Mercuric succinimide	O. H.	Greater	Less
Mercuric salicylate	M.	Less	Same
Mercurio-iodo-hemol	O. H.	Slightly less	Same
Mercuriol (Merek)	O. H.	Less	Slightly less
Mercury phenolphthalein	O. H.	Greater	Same
Mercury tetraiod phenolphthalein.....	O. H.	Same	Same
Fluorescein and mercuric chloride.....	O. H.	Less	Same
Trypan blue mercurous chloride.....	O. H.	Same	Same
Trypan blue mercuric chloride.....	O. H.	Same	Same
4-ortho-oxybenzylidene amino phenyl para mercuric acetate	O. H.	Less	Same
1-amino-2(p-naphthalin azo phenyl mercuric acetate) 5-sulphonic acid	O. H.	Less	Less
Methylene blue and mercuric chloride.....	O. H.	Much greater	Much less
Iod methylene blue and mercuric chloride.....	1305	Same	Same or less
Methylene green and mercuric chloride.....	O. H.	Same	Same
Diazo-amino methylene blue o-toluidin mercuric acetate	O. H.	Greater	Less
Diazo amino methylene blue from hydrate o-toluidin mercuric acetate	1305	Same	Same
Diazo amino methylene blue o-phenol mercuric chloride	1305	Same	Same
	1305	Greater	Less

Only with one set of animals treated with the double salt of methylene blue and mercuric chloride and with a set treated with diazo amino methylene blue o-phenol mercuric chloride has there been a similar beneficial effect on animals inoculated with a virulent strain, and with none of these drugs has the effect been sufficient so that we could speak of having really cured the disease. As is well known, the guinea-pig is far more susceptible to tuberculosis than is the human being and responds to infection with a more rapidly progressive and fatal disease than does man. Hence a treatment that would cure a tuber-

culous guinea-pig would presumably cure man more easily. A drug that has a decidedly beneficial effect in the guinea-pig would probably have a much greater effect in man. It may well be that the disease caused in guinea-pigs by a less virulent strain like the one described in this paper as the "Old Human" resembles far more nearly the disease in man than does the infection with the virulent strains (Miller's or 1305). However that may be, slight as seem the results reported in this paper, they seem sufficient to justify future work with mercurial compounds in the chemotherapy of experimental tuberculosis. Such work with many new organic compounds of mercury is already under way and will be reported at intervals, as results are obtained that seem worthy of presentation.

THE EFFECT OF BODY CELLS AND FLUIDS ON CERTAIN DYES

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It is a well-known fact that certain dyes are highly toxic to micro-organisms. This action is more or less specific in character so that although dianil blue is active against trypanosomes, it is almost without effect on *B. typhosus*. Gentian violet is germicidal to gram-positive bacteria. Also it must be borne in mind that a dye which is efficient in vitro is not of necessity of equal value in vivo. Chamberland,¹ working with oils, observed this difference in bactericidal action within and without the animal body and has been corroborated by numerous investigators. Test tube activity, however, is the best preliminary criterion thus far discovered for possible effectiveness in the body.

The usefulness of dyes for chemotherapeutic purposes is dependent on a number of processes that are of interest. These may be subdivided into (a) their germicidal value in vivo, (b) their effects on living tissues, (c) the processes by which they are removed from the circulation after introduction to it, and (d) the channels by which excretion takes place. These subjects should be considered before final judgment of the worth of a substance for purposes of treatment is rendered.

An endeavor has been made to learn whether any one of the available coloring matters is efficient for chemotherapeutic use in treatment of experimental infection by *B. typhosus* in rabbits. In searching for information in this field, experiments both in the test tube and in the animal, were carried out.

The particular scope of the work here outlined, which is one subdivision of that noted in the second paragraph, includes observations concerning the processes by which dyes are removed from the circulation after intravenous injection. The methods are those of the test tube rather than those pertaining to experiments made on the living animal since our knowledge of physiologic technic is not sufficiently advanced to allow the latter and more exact procedure to be used.

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¹ Ann. d. l'Inst. Past., 1887, 1, p. 152.

It was noted that while one dye may be eliminated in part through the bile, another may be found only in the urine. This of itself denotes a selective affinity in the first coloring matter for special cell structures which does not exist in the second dye. Again, one compound, as for example pyronine G, shows no visible signs of its presence in the body a few minutes after injection while proflavine stains the intercostal muscles for weeks. Fat soluble dyes like sudan III concentrate in the various storehouses of that tissue while new fast green 3 B tends to color connective tissue and parenchymatous structures. These examples suffice to indicate that decolorization of the dye in the serum follows its introduction and also that it appears to be possible that special cell structures are instrumental in separation of the coloring substance. Likewise the tissues or fluid most active may vary with the anilin derivative used.

Although coloring matters have found their principal medical use in bacteriologic technic in staining, for selective mediums and as trypanocides, Neisser and Wechsburg² availed themselves of methylene blue for the purpose of differentiating living from dead cells. Certain investigators have endeavored to indicate and to measure cell respiratory phenomena by the reactions of dyes. Evans and his collaborators,³ especially Schuleman,⁴ have studied vital staining and have shown that a stain in the colloidal state is toxic although when in solution it may be harmless. Likewise their more complete experiments prove that a colloidal stain is much less diffusible than one in which the particles are smaller. Evans has demonstrated that when trypan blue is administered to the circulation in tremendous doses, it is removed by a series of cell structures known as the macrophages.

Reference to Fay,⁵ Wahl and Attack,⁶ Bucherer or other standard texts on the chemistry of dyestuffs shows that coloring matters are built up from the leuco-base which is without tint and that intermediate to these there is another elementary material known as the color base. The distinction, at least as regards the triphenylmethane group, is one of progressive addition of oxygen to the molecule and the first two are colorless. Reduction of the dye in this rosanilin class of

² Ztschr. f. Hyg. u. Infektionskr., 1901, 36, p. 299.

³ Am. J. Phys., 1915, 37, p. 243.

⁴ Science, 1914, 39, p. 443.

⁵ Chemistry of Coal Tar Dyes, 1911.

⁶ Manufacture of Organic Dyestuffs, 1914.

⁷ Lehrbuch d. Farbenchemie, 1914.

compounds results in loss of color which, if the chemical changes have not proceeded too far, may in turn be restored by the introduction of hydrogen peroxide or bubbling oxygen.

The surmise stated in the fifth paragraph was followed and an endeavor was made to study some changes induced when suspensions of living cells and dilute dyes are brought together. It will be understood that the resulting effect on the dye itself rather than that on the cell was the goal. Little attention, therefore, was paid to vital staining phenomena.

This procedure was used: Cell suspensions, defibrinated blood, blood treated with sodium oxalate and two fluids, beef bile and serum were tested. The bile was obtained from a local slaughter house. The serum was from the horse. Because of the large amount of bacterial contamination in the bile when received at the laboratories, it was autoclaved at 15 pounds' pressure for 20 minutes. The serum was unaltered since it had been drawn aseptically at the Citter Laboratories in Berkeley and was furnished through the courtesy of that company. All other fluids and cell structures were from the rabbit.

Rabbit cell suspensions were made from organs removed immediately after the death of the animals, which in all cases had been killed by exsanguination. While the removal was not made under aseptic conditions, no gross contamination occurred. In some instances it was necessary to postpone use of materials until the following morning but in the meantime they were stored on ice in sterile dishes. The cells therefore were probably living at the time the suspension was made. Organs obtained were macerated in mortar with pestle in sterile physiologic salt solution. The mortar was used rather than a grinding machine because it was desired to procure an ultimate suspension as free from other cells, such as connective tissue, as possible. Red blood corpuscles (RBC) were prepared by centrifuging the defibrinated, sterile blood and then washing the sediment three times by whirling in sterile salt solution. The necessary mass was then added to fresh salt solution by means of a sterile pipet. The muscles utilized were the heavy ones from the region of the thigh.

The concentration of cells in the sodium chlorid solution was such that when settling had taken place after a few moments, the layer on the bottom of the tube in thickness was $\frac{1}{6}$ to $\frac{1}{7}$ of the total height of the column of liquid. Large particles were excluded by pouring the mixture into a conical glass and allowing them to settle out for a few seconds. Agglutination tubes were used in this series of experi-

ments and into each was poured from 1 to 1.5 c c of the suspended cell material. Sufficient dye solution was then added to each to give a decided tint after which they were shaken gently to give even mixing throughout the contents. The amount of dye in the tubes varied according to its color intensity but the limits were between 1:15,000 and 1:30,000. Finally, paraffin oil was overlaid in the tubes to exclude free circulation of oxygen after which incubation at 37 C. followed for 24 hours. When bile and serum were studied, the color intensity of the dye used was the same as that above noted.

At the end of the specified incubation period, observations were made using for purposes of comparison, when it seemed advisable, a check tube to which no dye had been added and which had received the same treatment in other respects as the remainder of the series. In some instances, sufficient material had collected against the side of the tube so that accurate observation of the remaining supernatant fluid could not be made and in such cases gentle centrifuging was resorted to in order to clarify it.

In making notes of the final condition of such preparations, it was evident that there were varying degrees of color intensity remaining in the supernatant liquid and that many preparations yielded cell sediments which were heavily colored while others retained their original and natural shade. Such color as they had taken up from the dyed suspension had been lost. Certain tubes resulted in entire loss of color both in the supernatant and in the cell layer at the bottom showing that not only had all the dye added been removed, but also that it had been destroyed. In a few instances, as with certain tubes containing methylene blue, the subsequent addition of hydrogen peroxide caused a return of the color with its former intensity, thus proving that in these samples the dye had been broken down to the leuko-base. In instances in which the color was not brought back by this reagent, it seems fair to assume that the destruction had progressed to some point below that of the leuko-base. In addition, microscopic examination was made of the detritus remaining at the bottom of the tubes and in a majority of instances no staining of the cells themselves could be demonstrated. On the other hand, clouds of stain could be seen dimly in masses of cell structures although no internal absorption of the dyestuff was evident either in nucleus or in cytoplasm. This may indicate that in these samples the stain had been taken up on the surfaces of the cells and later destroyed at that site by physical or chemical means or by both. It is quite possible that this action

was effected through the agency of cell substance by adsorption which probably means the formation of ephemeral and unstable combinations at the surface.

The results of this series of experiments with dyes in contact with living cells in vitro is given in the table. "Supernatant" refers to results of observations made on the overlying fluid. The following abbreviations and characters are used for ease in tabulation: "R B C" means red blood corpuscles; "Des." (destroyed) indicates no color remaining; "Tr." means trace; "N C" (no change) shows that no appreciable color intensity was lost in the supernatant. "Fair" is adopted as meaning some degree between no change in color and a trace. The term "S D.," used in connection with brilliant green and bile, refers to slight darkening which was the outcome in that special instance. "Yel." means yellow.

THE EFFECTS OF CERTAIN CELLS AND FLUIDS ON DYES AS SHOWN BY CHANGES
IN THE SUPERNATANT FLUID

Stains	Bile	Serum	Red Blood Cells	Defib- rinated Blood	Oxa- late Blood	Brain	Mus- cle	Lung	Mar- row	Liver	Spleen	Kid- ney
Acriflavine.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
Basic fuchsin.....	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Fair	Fair	N.C.	Des.
Benzoazurin.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	Des.	Des.	N.C.	Fair
Brilliant green.....	S.D.	N.C.	N.C.*	Tr.	Tr.	N.C.	Des.	N.C.	Des.	Tr.	Tr.	Tr.
Congo red.....	N.C.	N.C.	N.C.	N.C.	N.C.	Fair	Tr.	Des.	Fair	Fair
Corallin.....	N.C.	Fair	Tr.	Tr.	N.C.	Des.	Des.	Des.	Des.	Des.
Crystal violet.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Tr.	Des.	Des.	Tr.
Cyanin B.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	N.C.	Tr.	Des.	N.C.
Erioglaucin A.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Fair	Tr.	N.C.	Tr.
Malachite green...	N.C.	Tr.	N.C.*	N.C.	Br'wn	N.C.	Des.	N.C.	Tr.	Des.	Des.	Tr.
Methylene blue....	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Des.	Fair	Fair	Des.	Des.	Des.
Methyl violet 5B...	N.C.	N.C.	Tr.	N.C.	N.C.	N.C.	Tr.	Des.	Fair	Fair
Neutral red.....	N.C.	Tr.	N.C.	Fair	N.C.	Yel.	Yel.	Tr.	Yel.
New fast green, 3B	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Tr.	Des.	Fair	Tr.
Oxamine violet....	N.C.	N.C.	N.C.	N.C.	N.C.	Fair	N.C.	Des.	Tr.	Tr.
Proflavine.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
Pyronine G.....	Fair	Tr.	N.C.	N.C.	Tr.	Tr.	Tr.	Des.	Des.
Safranin.....	N.C.	N.C.	N.C.	N.C.	N.C.	Fair	Tr.	Fair	Fair	Tr.
Sauregrün.....	N.C.	N.C.	N.C.*	N.C.	N.C.	N.C.	Des.	Tr.	Tr.	Des.	Fair	Des.
Setocyanin.....	N.C.	N.C.	Tr.	N.C.	N.C.	N.C.	Des.	Fair	Des.	Tr.	Des.	Tr.
Spiller's purple....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Fair	Tr.	Fair	Fair	Fair
Trypan blue.....	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Des.	N.C.	Des.	Des.	Tr.	Des.

* Cells disintegrated.

It should be considered that it is not impossible that the changes noted may be connected with cellular respirational function of interchange of gases. The mechanics of removal, however, have not been explained.

SUMMARY

On reading the table it will become evident that when brought into contact with serum, bile and brain, little change was wrought in any

of the coloring matters. On the other hand, certain secreting and filtering tissues, such as liver, kidney, bone marrow and spleen, showed a high degree of activity. Lung and muscle occupy an intermediate position. Certain dyes such as acriflavine and proflavine showed no change throughout. Other stains varied in outcome according to the particular cell structure with which they were placed in contact. Some dyes were reduced to the leuco-base as methylene blue many times and as trypan blue once. Others were changed into products further removed from the original molecular structure. It seems sufficiently conservative to offer the following deductions:

Dyes are removed from the circulation through the agency of cell structures. Tissue cells rather than blood corpuscles are instrumental in this respect since neither serum, washed corpuscles, defibrinated blood nor blood treated with sodium oxalate is active as a decolorizing agent *in vitro*.

The particular tissue instrumental in this removal varies with the dye in question.

Separation of the dye from its solution in the serum is brought about by adsorption on cell surfaces with no vital staining evident at concentrations used.

Stains thus removed by adsorption are destroyed.

This molecular destruction of the dye continues to the production of the leuco-base in a few instances only, while in a great majority it extends to the formation of products further removed from the original chemical structure.

IMPROVED TECHNIC FOR THE MICRO OR LITTLE PLATE METHOD OF COUNTING BACTERIA IN MILK

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INTRODUCTION

I have previously described a method for counting bacteria in milk and other richly seed material.¹ It is possible by means of this method to get counts in a few hours. It has been found also, by myself² and Simmons,³ that the count obtained is reasonably close to that given by the Koch plate method.

In the last few years the method has been put to practical test and, furthermore, since the technic has been improved in several important particulars, it seems necessary to give a description of the method in detail.

When it was first published the impression was conveyed to some, at least, that it would be necessary to limit the period of incubation to a few hours and this was interpreted as a disadvantage because of the necessity of coming to the laboratory "out of hours" in order to take the plates out of the incubator and dry them. In reality, however, the incubation period can be extended to 16 or even 24 hours without affecting the count. It is thus possible to make the cultures one day and leave them in the incubator until a convenient time the next day.

By using a smaller pipet, it is possible to measure the sample directly onto the slide; this shortens the method, obviates the necessity of diluting the sample, economizes the medium and materially simplifies the method.

The special apparatus needed has been standardized and satisfactory types put in commercial form. A portable outfit, in a combination

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¹ Jour. Am. Med. Assn., 1916, 66, p. 889.

² Jour. Infect. Dis., 1916, p. 237; Jour. Bacteriol., 1917, 2, p. 567.

³ Jour. Infect. Dis., 1919, 24, p. 322.

carrying case and incubator, has been devised, which ought to be of real value in the solution of field problems.

APPARATUS NEEDED

Glass Slides.—The apparatus needed consists, first of all, of glass slides. These must have marked off on them a definite area, of 4 square cm. (fig 1). It would seem best to have such areas permanently fixed, and slides can be purchased so marked.⁴ Since these are quite expensive, most workers will no doubt continue to use ordinary microscopic glass slides (1 by 3 inches) marked with a wax pencil. This grease border line has a distinct advantage since it causes the milk and agar to flow back and so the marked off areas are not overrun in making the "little plates." In order to have the areas accurate, it is best to use some mechanical means for the purpose of marking them. The apparatus illustrated in fig. 2 is suggested as satisfactory, although it is possible to mark off the areas fairly accurately by tracing the lines over a paper pattern. It is economical to mark off two areas on each slide.

Forceps.—For handling the slides a good forceps is needed. Fig. 3 represents a desired form.

Warm Table.—In order to prevent the hardening of the agar before it is thoroughly mixed with the milk, it is necessary to spread the little plates on a "warm table." A convenient form is a metal box surrounded with asbestos on all sides except the top and bottom. In it warm water (45 C.) is placed. At one end, tubulations to hold two test tubes of medium are inserted at an angle of about 45 degrees (fig. 4).

Special Pipets.—One pipet about 8 in. long made of capillary tubing is suggested. The tube should be sufficiently small so that about one-half inch will hold one hundredth of a c c and a twentieth of a c c would equal about $2\frac{1}{2}$ in. If the pipet is marked in 0.01 c c, the same pipet can be used to make a direct count (Breed), as well as the little plate count (fig. 5). Such pipets are now stocked by most apparatus firms as "serological pipets."

Moist Chamber Cabinet.—This is shown in fig. 6. It is simply a rack to hold 48 slides and is provided with a space for water in the bottom and room on the sides for the moisture to circulate. The rack is made removable. This makes it possible to prepare all of the slides for microscopic examination without handling them individually.

Hot Plate or Drying Oven.—It is necessary to dry the little plates rapidly, and this is best done by keeping them for about 5 minutes just below the boiling point of water. When the plates are handled individually, a metal box is used with a flat and level top in which water is kept at or near the boiling point. Where the slides are to be dried in the rack, an oven is necessary and an electric one is very satisfactory.

Staining Outfit.—For the purpose of staining the individual slides a staining jar is needed. A good form is the Coplin jar. A tumbler or larger jar is needed for the wash water. In case the whole rack of slides is to be stained at once a special container for the rack is needed.

⁴ Central Scientific Co., Chicago, Ill.

MATERIAL NEEDED

Culture Medium.—Ordinary nutrient agar is used. It is made and sterilized in the usual way (standard methods Am. P. H. Assn.). One test tube of medium (5 cc) will be sufficient for about 50 samples.

Acetic Acid Solution.—A 10% solution of glacial acetic acid in 95% alcohol.

Stains.—Methylene blue and thionine are both satisfactory. Loeffler's methylene blue diluted with three times its volume of distilled water has been used most.

It is also possible to make a satisfactory stain by taking 10 cc of a saturated alcohol solution of methylene blue to 400 cc of distilled water.

The formula for the thionine stain is: thionine blue, 1 gm.; carbolic acid, $2\frac{1}{2}$ gm., and distilled water, 400 cc; filter. To this is added 5% of glacial acetic acid. The slides are put in this stain without preliminary treatment with acid alcohol. We have found this the most satisfactory stain.

METHOD IN DETAIL

Melting Agar.—A tube of agar is melted by placing it in boiling water. It is then placed in one of the tubulations on the warm table. The cotton plug is removed and a pipet which will deliver a small drop (about 0.05 cc) is placed in it.

Filling the Warm Table.—While the agar is melting, the warm table should be filled with water that has a temperature of about 45 C. It is necessary to note the temperature occasionally. If it gets down to 40 C., the agar is likely to harden, and if it gets much above 45 C., it is probable that some of the milk bacteria will be killed or at least devitalized to such an extent that they will not form colonies within the usual period of incubation.

Mixing the Milk.—The bottle or sample should be shaken at least 25 times by tipping it from end to end slowly enough to avoid the formation of a foam, since air bubbles would interfere with the accurate measurement of the sample in the pipet.

Sterilizing the Glass Slides.—The glass slides, which have been properly marked, are sterilized in the direct flame. In order to do this, they are taken up in the forceps and passed through the flame, about three times, marked surface down, and once on the other side. About a half dozen are thus sterilized and placed on the warm table.

Pipetting the Sample.—A special pipet has already been described. Approximate results can be obtained with any pipet that will measure a small quantity, as 0.05 cc, but since the accuracy of the count depends on the correct measurement of the sample, the construction and use of the pipet becomes a matter of great importance. It is necessary to use a pipet of small bore in order to measure accurately. This necessitates the use of heavy walled tubing. The end must be pointed, otherwise too much milk clings to the outside.

It is important, in using a pipet, not to dip it into the milk any farther than is necessary to avoid taking in air bubbles. The reason for this is that milk clings to the outside of the pipet and will run down and swell the sample. In any case, the tip of the pipet after it is filled should be brought in contact with the inside of the bottle above the milk to drain off the extra milk, or wiped with a piece of sterile filter paper or a sterile towel. Five one-

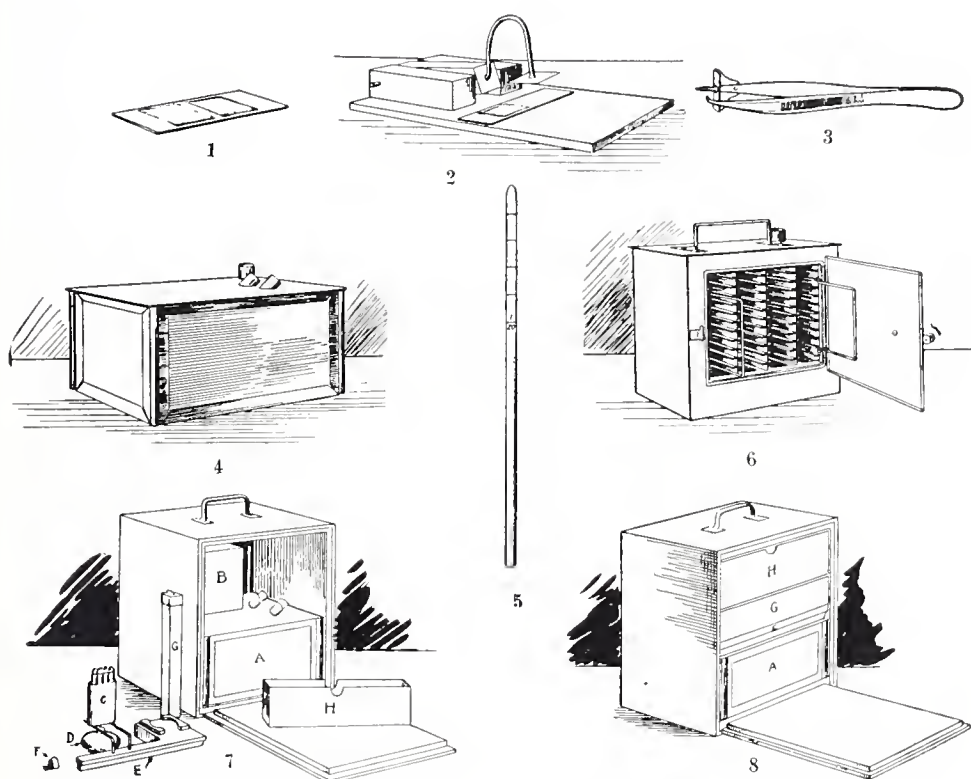


Fig. 1.—Microscopic glass slide with two areas of 4 sq. cm. each marked off with a wax pencil.

Fig. 2.—A guide for marking the slides.

Fig. 3.—Forceps provided with a stop which makes it possible to handle slides easily.

Fig. 4.—A "warm table." This is an asbestos covered metal box which holds about 2 liters of water and is used to keep the slides warm while the milk and agar are being spread to form the "little plates." It also serves to keep the tube of liquefied agar from solidifying. It has 3 tabulations, 2 at an angle for holding tubes of medium and the other for a thermometer.

Fig. 5.—A serologic pipet which delivers 0.01 c c; 0.05 is the amount of milk usually used.

Fig. 6.—A "moist chamber cabinet." The "little plates" are kept in this while they are in the incubator. Water in the bottom keeps a moist atmosphere. The rack is made removable so that all of the slides can be dried (in an oven), fixed and stained altogether, and thus avoiding the handling of each slide separately.

Fig. 7.—Field Outfit. The case is wood and is lined with an insulating material and when closed forms an incubator on the principle of a "fireless cooker." A is the "warm table" (fig. 4) which is filled with water 42-43 C. B is the "moist Chamber cabinet" (fig. 6) and contains the little plates. C is a cup which holds the agar tubes and in which they may be melted over the alcohol lamp D. E is a support for the cup and also holds the various articles in place when the case is packed. F is the cap to the alcohol lamp. G is the pipet case and H is a box in which are placed the glass slides, platinum loops, etc.

Fig. 8.—Carrying case packed ready to be closed. It then becomes an incubator. The outfit holds all the material necessary for making an analysis of 48 samples in duplicate.

hundredths ($\frac{1}{20}$) c.c. of the sample of milk is placed in each area marked off on the glass slides, i. e., two equal portions from each sample are put in different squares on the same slide.

The milk portions should be put on only a few slides at once because they begin to dry before the agar is added to them, if allowed to stand on the warm table.

Adding Agar and Spreading Film.—As soon as possible after the milk has been put on the glass slides, a drop of agar should be added to each. An exact amount is not absolutely demanded, but it should be approximately 0.05 c.c., and pipets should be selected that will deliver that amount in one or two drops. Practically the same amount should be used each time, otherwise the concentration of the agar will vary, and consequently, its consistency. This variation does not appear to affect the number of the colonies, but it influences their appearance.

The milk and agar must be thoroughly mixed and spread evenly over the 4 sq. cm. area. This is exactly done by means of a sterile platinum loop.

In order to spread the films as quickly as possible, it is desirable to have two loops and some arrangement for holding them in a flame so that one can be sterilizing while the other is in use. If the slides are properly heated at the time of sterilization, the film is easily spread and can be made to cover evenly the entire area. When the film is properly spread, it must be hardened. If the shelves in the moist chamber cabinet hold the slides in a level position, they can be put directly there, otherwise it will be necessary to arrange a place to harden them. If the work table is level, they can be placed there under a cover which will protect them from air contamination. When the film is hard, they are ready for the moist chamber and incubation.

Moist Chamber Cabinet.—It is necessary to keep the little plates from drying down during incubation. For a few slides and as a makeshift Petri dishes can be used. In this case the Petri dish has the bottom covered with wet filter paper; on this are laid two glass rods or match sticks, and on these the slides may be placed, two to a dish. It is more convenient, however, to have the special apparatus designed for the purpose and called a "moist chamber cabinet," which has been described. When a considerable number of milk samples are to be examined, the cabinet is essential. It should be thoroughly cleaned from time to time to prevent contamination by such organisms as produce spreaders on ordinary plates. It is quite sufficient to wash the cabinet in hot water or boil a little water in the bottom of it by placing it over a flame. It should, of course, always be cooled before use, and when in use it should always have at least enough water in it to cover the bottom in order that the air may be kept moist.

Incubation.—The little plates are best incubated at a temperature of 37.5 C. The moist chamber cabinet is put in an ordinary incubator, although a heavily insulated case, together with a source of heat, similar in principle to a fireless cooker, can be used. The possibilities of this are discussed later.

The period of incubation varies. If it is necessary to get results at the earliest possible moment, even so short a period of incubation as 4 hours will be satisfactory if the bacteria are actively growing in the milk at the time of sampling. If the milk has come from cold storage, has been pasteurized or is fresh and low in bacteria, 7 or 8 hours are necessary; if, on the other hand,

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there is no particular hurry about the results, the plates can be left in the incubator from one day to the next, i. e., 15 or 16 hours.

They can also be incubated at room temperature (20 C.). In this case it seems necessary to leave them for at least 24 hours. Even then the count seems lower than that obtained by incubating at 37 C., although this has not been fully established.

Drying Films.—When the little plates are taken from the incubator, they should be dried at once. If they are allowed to dry at the room or incubator temperature, the films have a tendency to crack and to peel off or loosen in the staining process. It is best to dry the films down rapidly by putting them on a hot plate or in a drying oven at a temperature slightly below that of boiling water.

Recent trials indicate that it is possible also to put the wet films directly in the acetic acid alcohol or even the stain when it contains the acetic acid.

Staining.—It is possible to stain the films so that the colonies are deeply colored, while the medium is almost wholly without color. The success of this process depends to a considerable extent on the treatment that the films receive before they are put in the stain. They must be properly dried as already indicated. They are then put in the acetic acid solution. This acts quickly but does not overact; hence, it should be applied for about one minute, but no harm is done if the exposure is a long one. The purpose of the acetic acid is to keep the agar from taking the stain. Any of the stains mentioned are satisfactory, and the time of exposure is about 2 minutes, although a longer exposure is not harmful. The thionine gives excellent results. The background is clearer than with methylene blue.

The slides should be thoroughly washed in tap water. When stained, the preparation should be dried, and for this purpose a drying plate is convenient; a temperature just below 100 C. is best.

Dilution of Heavily Contaminated Samples.—If a milk is likely to have more than one million bacteria per cc, it should be diluted before a little plate is made. When water blanks are used, as is the common practice with the Koch plate method, comparatively little milk is taken over into the culture. If the same method were followed with the little plates, the food conditions would be quite different in the direct plates and those diluted with water. In order, therefore, to have comparable conditions in all of the little plates, it is advised that sterile milk be used for dilution purposes instead of water. It is necessary to have tubes of sterile milk containing 9 cc. To these is added 1 cc of the milk to be counted, and then after thorough mixing the diluted sample is used in exactly the same way as the ordinary samples.

A simpler way, however, is recommended, especially for field work. This was suggested by W. D. Dotterer of the Bowman Dairy Co. A small fraction of a cc of the milk to be analyzed is used, 0.01 or 0.02 cc and to this, on the slide, a drop of sterile milk is added from a tube which is kept in the second tubulation on the warm table. To this is added the agar, and the whole is spread with perhaps more than usual care.

Counting Colonies.—The counting is done under a compound microscope. If the colonies are large and few in number, the low power can be used, and this is best. If the colonies are small or numerous, the higher powers must be used. With the immersion objective, the oil is put on the dried agar. There

is no objection to mounting them in balsam and using a cover glass. This is best if the high dry powers are to be used. At first it seemed necessary to count at least 20 fields widely distributed over the film in order to get a fair average. For this a mechanical stage was recommended. Further experience leads to the conclusion that, ordinarily at least, it is only necessary to count 5 fields. In this case the slide is put under the microscope and moved about to get a general idea of the distribution and then 5 representative fields are counted. In counting, several things should be kept in mind. First, all of the colonies within the fields should be counted and only half of those that touch the edge, say those on the right half of the periphery, while those touching the left edge of the field should not be counted. Second, with the highest powers single bacteria or small groups should not be counted, on those plates that have been incubated 7 or 8 hours or more. In plates incubated as long as that, practically all of the original live bacteria have grown into definite colonies. Here as elsewhere, experience and good judgment are necessary to get consistent results.

Calculating the Number of Bacteria per Cubic Centimeter of Milk.—Having determined the number of colonies in a given number of fields of the microscope, or the number in an average field, it is necessary to multiply that number by some factor which will convert the figures representing the number of colonies per field of the microscope into figures which will represent the total number of bacteria per c c of the milk.

In order to do this it is only necessary to determine the area of the microscopic field, for the value of all of the other factors is known, i. e., the area of the little plates (4 sq. cm.) and the amount of milk used (0.05 c c, 0.005 c c, etc.).

The following formula is used:

$$\frac{\left. \begin{array}{l} \text{The number of bacteria} \\ \text{per c c of milk} \end{array} \right\} \times \frac{\text{No. colonies counted}}{\text{No. fields counted}}}{\left. \begin{array}{l} \text{Area of little plates} \\ \text{Area of the microscopic field} \end{array} \right\} \times (\text{or microscopic factor})} \times$$

Reciprocal of the dilution of the milk.

Let:

Number of bacteria per c c of milk = X

Number of colonies counted divided by the number of fields counted, i. e., the average number of bacteria in a microscopic field = C.

The area of the little plates (4 sq. cm. or 200 sq. mm.), divided by the area of the microscopic field or microscopic factor = M. The reciprocal of the dilution of the milk = D.

Then, $X = C \times M \times D$. The only unknown value in the second half of the equation is the area of the microscopic field. This must be determined not only for each microscope, but also for each combination of lenses.

The area of a disk equals the radius squared times 3.14159 (π), or the diameter squared times 0.7854 ($\pi/4$).

In order to determine the diameter of the field of a microscope a stage micrometer is necessary. This should be ruled to 0.1 and 0.01 mm.

By regulating the length of the tube, the size of the field may be varied. It would seem desirable, however, to use the ordinary tube length, otherwise

LITTLE PLATE METHOD OF COUNTING BACTERIA IN MILK 183

there is always danger that the tube may not be properly drawn out and the count thus affected.

For the convenience of those who may not have a stage micrometer at hand, the following table is included, which gives the approximate diameters and the areas of the microscopic fields of an American microscope. This will give a value which will serve until there is an opportunity to have the diameter of the particular microscope carefully determined.

DIAMETER OF MICROSCOPE FIELDS WITH COMMON COMBINATION OF LENSES AND THE TUBE PUSHED IN (160) MM.

Objective (Equivalent Focus)	Ocular	Diameter of Field	Area of Field
$\frac{2}{3}$ inch or 16 mm.....	10 x	1.55 mm.	approximately 2 sq. mm.
$\frac{1}{6}$ inch or 4 mm.....	10 x	0.31 mm.	approximately 0.08 sq. mm.
$\frac{1}{12}$ inch or 2 mm.....	10 x	1.555 mm.	approximately 0.02 sq. mm.

In order to make correction for eyepieces of different magnifying power, multiply the area given above for the 10 x ocular by 1.4, for an 8 x by 1.5, for a 7.5 x, by 2 for a 5 x and 2.5 for a 4 x. With a 16 mm. objective and a 10 x ocular the area of the microscopic field is approximately 2 sq. mm. In order to get the microscopic factor, the area of the little plate (400 sq. mm.) is divided by the area of the microscopic field (2 sq. mm.). This gives 200. This figure multiplied by the denominator of the fraction of a c c of the milk used in making the little plate gives the factor necessary to convert the colonies in the field of the microscope into bacteria per c c of milk. The usual dilution is $\frac{1}{20}$ c c; this means that each colony in the field of the microscope represents 4,000 bacteria per c c.

The same figuring would show us that each colony under a 4 mm. objective would mean 100,000 bacteria per c c of milk and one colony under the oil immersion would mean 400,000 bacteria per c c of milk.

FIELD OUTFIT

Because of the small amount of material required to make the cultures and its simplicity, this method ought to be of special value in field work.

A carrying case is here described which may also serve as an incubator. It is shown packed in fig. 8 and its contents are exhibited in fig. 7. It is a case about $13\frac{3}{4}$ inches long, $13\frac{1}{2}$ inches high and $9\frac{3}{4}$ inches wide. It is lined with an insulating material. In the particular case figured, the insulating material was made of flax straw. The important dimensions are the inside ones, $10\frac{3}{4}$ inches long, 11 inches high and $6\frac{1}{8}$ inches wide. The door is arranged to close tightly; this is accomplished by using a thick felt strip in the jam. In this case are fitted the warm table A; the moist chamber cabinet B; a support for holding the needles and a cup for melting the agar over a flame, E; an alcohol lamp D; a cup for holding the medium and melting it C; a pipet case G; and a box for such articles as glass slides, needles, forceps, wax pencil, etc. The case is small and packed with material necessary for duplicate plates of 48 different samples. The weight is only about twenty pounds.

If one plate only were made, 96 samples could be analyzed. If the trip were to be long, a large number of samples could be analyzed by taking

extra slides, pipets and medium, provided one set of slides were incubated and dried down before the next set of plates were made. The dried plates keep indefinitely, either stained or unstained, in slide boxes.

To use the carrying case as an incubator, it is only necessary to fill the warm table with water at about 43 C., pack and close the case. When the case is kept at ordinary room temperature, the heat of the water will bring the chamber up to about that of blood heat and hold it above 30 C. for at least 12 hours. In this case the colonies grow out satisfactorily in from 8 to 16 hours.

A STUDY OF THE THRUSH PARASITE

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The organism of thrush was discovered by Berg¹ in 1839. Robin, quoted by Plaut,² in 1847 named the organism *Oidium albicans*, a name it has held most constantly up to the present day. The discovery led immediately to botanical studies of the organism. Many works have been published, but unfortunately these are lacking in agreement. A glance into bacteriological textbooks reveals but meager descriptions of this organism. Points on which reports vary most widely are its morphology and botanical position and the unity or plurality of species.

Microscopically the thrush organism may appear in yeast-like or filamentous form. The filaments are simple or branched, definitely septate, showing thick cross walls. The cells contain protoplasm, vacuoles, granules, a nucleus and fat globules. The contents in young cultures are homogeneous, in older cultures vacuolated. Simple filaments may give rise to short globular buds at their sides. These buds may in turn elongate and form branches, and these branches may again bud and again branch out. This often leads to such an interlacing of filaments that it is impossible to follow any one.

The formation of filaments, according to Linossier and Roux,³ may take place by two distinct processes. Sometimes a bud appears on the cell which separates off immediately by a manifest septum. It does not remain round but lengthens, becoming the segment of a hypha, and reproduces other segments by a similar mechanism. Or the initial yeast cell may push out a prolongation like a finger of a glove, which is not separated by a septum and retains the same protoplasm.

The formation of filaments, according to Linossier and Roux,³ may They bud actively. As a rule the buds separate on attaining mature size and form new daughter cells, but sometimes they remain attached, presenting the appearance of beads or bouquets.

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¹ Ueber die Schwämmchen bei Kindern, 1842.

² Kolle und Wassermann Handbuch der path. Mikroorg., 1913, 5, p. 42. Deutsch. med. Wehnschr., 1894, 20, p. 920.

³ Compt. rend. de l'acad. d. sc., 1889, 109, p. 752. Arch. de med. exp. et d'anat. path., 1890, 2, p. 62.

In the false membranes, or "plaques," found in the mouth, the yeast-like cells are attached to the filaments, or free. The attachments are lateral or terminal and so resemble conidia that many authors use the term "conidia" in speaking of the globular forms.

In cultures the organism appears either in the pure yeast form, or in the pure mycelial form (though this is rare) and frequently in both forms. The appearance of either or both of these forms depends on the composition of the medium and other factors which will be discussed later.

The first obstacle which presents itself in the study of the parasite is that of classification and systematic position. Since its discovery the organism has been named as follows: *Sporotrichum* by Gruby, quoted by Plaut, *Stemphylium polymorphum* by Hallier,⁴ *Mycoderma vini* by Grawitz,⁵ *Dematium albicans* by Laurent,⁶ *Mucor* by Linossier and Roux,³ *Syringospora* by Quinquaud,² quoted by Plaut, *Saccharomyces* by Guidi, Reess, quoted by Plaut,² Fisher and Brebeck,⁷ and Audry,⁸ *Endomyces albicans* by Vuillemin,⁹ and *Oidium lactis albicans* by Robin, quoted by Plaut.²

But, as Plaut² points out, Gruby named it *Sporotrichum* because of the side ectospores; but many fungi form such ectospores. *Stemphylium* has brown or black spores and in no way resembles the thrush parasite. *Mycoderma vini* is out of the question because it is non-pathogenic. The thrush parasite differs from *Dematium* in the manner of spore formation. The mere finding of chlamydospores in this organism is not sufficient to name it *Mucor*, for chlamydospores are common to many fungi. The distinct septation of the thrush filaments separates it from *Phycomycetes*. Its action toward chemicals, its pleomorphic forms, and the presence of chlamydospores removes it from *Saccharomycetes*.

The problem narrows down to the question of classifying the organism in the genus *Endomyces* or the genus *Oidium*.

According to Stevens,¹⁰ the genus *Endomyces* belongs to the family *Endomycetaceae*, order *Saccharomycetales*. The characteristic features of the family are a mycelium usually well developed, producing

⁴ Botan. Ztg., 1865, 23, p. 253.

⁵ Virch. Arch., 1886, 70, p. 546.

⁶ Bull. Soc. belge de micr., 1890, 6, p. 14.

⁷ Zur Morphologie, Biologie und Systematik der Kahmpilze, der Monilia Candida und des Soorerregers, 1894.

⁸ Rev. de med., 1887, 7, p. 586.

⁹ Revue mycologique, 1899, 21.

¹⁰ Fungi Which Cause Plant Disease, 1913.

a luxuriant growth, multiseptate; asci borne singly on branches or intercalary, 4-8 spored, and unicellular conidia produced apically. The genus *Endomyces* is characterized by asci, 4 spored. The genus *Oidium* belongs to the family Oösporeae, order Moniliales, of Fungi imperfecti, since it produces no ascospores.

If the presence of asci and ascospores as mentioned by Fisher and Brebeck can be verified, we are justified in classifying the thrush parasite with *Endomyces*. The absence of these structures leaves for the organism no other genus but that of *Oidium*.

Three types of spores have been described as occurring in the thrush parasite, namely, conidia, chlamydospores, and ascospores.

The yeast-like globules or conidia forms are surrounded by a membrane slightly thinner than that of a pure yeast. This membrane gives no cellulose reaction. Within the membrane are cytoplasm and nucleus. The cytoplasm contains granules and vacuoles. Within the vacuoles can be seen the little dancing granules which are so characteristic of yeast cells. In young cultures the protoplasm is hyaline and homogeneous. In older ones it becomes vacuolated and granular. It takes the basic aniline dyes and retains Gram's stain. The nucleus is small but can be distinctly seen with Heidenhain's iron hematoxylin. It is surprising to read Linossier's and Roux's³ statements that they could detect no nucleus.

According to Linossier and Roux,³ these globular cells are not spores but the vegetative phase of the plant, which can adapt themselves to all sorts of mediums. Vuillemin⁹ believes them to have the functions of both spores and vegetative cells. Daïreuva¹¹ states that the "conidia" bud when conditions are favorable; when unfavorable these are able to resist drying, abnormal temperature and lack of food by changing into mycelium.

Chlamydospores are described by Linossier and Roux³ as follows:

The form is characterized by certain filaments at the extremities of which are spherical cells. These cells or chlamydospores are larger than the conidia, sizes ranging from 9-24 mikrons. They may also be found between two segments. They are spherical and their protoplasm is more refringent and granular than that of the "conidia" or filaments. Their membrane is thicker. The protoplasm in the chlamydospores is at first finely granular and but slightly refractile; later it becomes coarsely granular. The granules are either arranged like a necklace or bunched, surrounding a central hyaline globule. The surrounding membrane of the terminal cell thickens and become glassy. On squeezing, it always opens at the same point by a V-shaped rent through which the granules with the central globule escape. Preceding this process glycogen accumulates in the preterminal cells, as shown by staining with iodine. These globules, as they escape, remain indefinitely in the media without further change, but have been made to germinate on raw cherries. They are believed to be chlamydospores which germinate in order that the parasite may develop in a new habitat.

The medium in which, according to these authors, the chlamydospores appear constantly is a liquid, each liter of which contains saccharose, 20 gm.; ammonium tartrate, 10 gm.; potassium phosphate, 1 gm.; magnesium sulphate, $\frac{2}{10}$ gm., and calcium chloride, $\frac{1}{10}$ gm. This medium was used for many of my studies.

Plaut² considered chlamydospores as involution forms of thrush. Burchardt (quoted by Plaut²) described "capsules" which he found in emulsified false membrane. He described them as being round, $\frac{1}{50}$ - $\frac{1}{12}$ mm. in diameter, double contoured and full of small spores. He considered them to be sporangia. Hausman (quoted by Plaut²), and Hallier⁴ also mentioned capsules which they called sporangia. Grasset (quoted by Plaut²), found chlamydospores in old broth cultures. Charrin and Ostrowsky¹² found some in dextrose broth cultures. Hickel¹³ described them as round cells three times the size of the ordinary cells, full of reserve food and surrounded with a thick refractile membrane. Vuillemin⁹ and Daireuva¹¹ have found them in old cultures and believe that they arise when conditions are unfavorable, such as lack of nutrition, presence of bacteria, or chemical influences. They observed their germination on transplanting them from beets to broth. I found them in Linsier's medium in two of my strains.

Vuillemin⁹ and Fischer and Brebeck⁷ have described ascospores. Vuillemin states that they are numerous in old cultures on various mediums and that asci 4-5 mikrons in diameter contain 4 ascospores.

Fischer and Brebeck found them in 5 cases of thrush. The strains formed a pellicle on milk; 14-day old cultures of these showed endospores. They also isolated a nonpellicle forming kind from a sixth case of thrush, but in this case they could find no endospores.

In infected tissue both "conidia," or spherical cells, and mycelium can be seen. In artificial medium we find the globular form alone under certain conditions, and obtain the mycelial or filamentous form under other conditions. All of the authors except Stumpf¹⁴ agree on the existence of a globular, as well as a filamentous form, but they do not all agree as to where and why each form appears.

Grawitz⁵ used a liquid medium made by adding to dextrose solutions ammonium tartrate and 2% of a mineral salt obtained from ashes. He used various concentrations of sugar and claimed that the greater the concentration of sugar in the medium the more the organism takes on the yeast form. Plaut² stated that he obtained mycelium in sugar-free nitrogenous medium, and yeasts in rich sugar medium. Audry⁸ believed that solid mediums cause the growth of the yeast form while liquid mediums cause the growth of the mycelial form. He planted the organism on lemons and obtained the pure globular forms, some free and some attached like pearls on a string. In broth, he said the round cells became oval, elongated and attached. Some gave rise to long filaments which bulged at one end. This bulging part pediculated in some cases and formed small adherent cells. The filaments were septate and rounded at their extremities.

Nearly all the authors are in agreement that yeast-like cells alone appear on the surface of solid mediums while mycelium may develop in some liquid mediums. The most elaborate research on this aspect has been made by

¹¹ Recherches sur le champignon du muguet et son pouvoir pathogene, 1899.

¹² Comptes rend. Soc. de biol., 1896, 48, p. 743.

¹³ 115, p. 159.

¹⁴ Sitzungsber. d. math.-naturwiss. Klasse der Akad. d. Wissensch. in Wien, 1907, Pt. 1.

¹⁵ Aerzt. Intelligenzbl., 1885, 32, p. 627.

Linossier and Roux,³ who after a thorough study of the organism on a variety of mediums arrived at the theory that "the complexity of form of the thrush organism is proportional to the increase in the molecular weight of the food elements in the medium." As a proof of their theory they cite the following experiment:

They planted the organism in a mineral liquid consisting of water 1,000 c.c., potassium phosphate 0.75 gm., magnesium sulphate 0.05 gm., ferrous sulphate 0.02 gm., zinc sulphate 0.02 gm., a trace of sodium silicate and ammonium sulphate 1 gm., and to this were added carbohydrates of various molecular weights. In the mediums of low molecular weight, such as lactose, glucose or glycerol, they obtained only yeast forms. In mediums of high molecular weight, such as dextrin or gum arabic, they obtained long abundant filaments.

They do, however, admit that this rule is not without exceptions; for they obtained filaments in acid, alkaline and nitrogenous mediums of low food value, and under conditions which were unfavorable, such as lack of food. They explained the presence of yeast-like cells on the surface of their solid mediums, especially those of high molecular weight, by saying that cells separated from the medium by other cells receive that medium by diffusion; only simple foods are diffusible, and therefore the organisms grow in the simple or yeast-like form. Cells directly in contact with the medium, namely, in a liquid medium, develop at its expense and consequently grow in mycelial form.

Hickel¹³ accepted Linossier and Roux's theory as "to the complexity of form in proportion to increase of food value," and carried out a similar experiment. He used a medium containing 500 gm. of water, 0.25 gm. of magnesium sulphate, 0.25 gm. of potassium phosphate, a trace of iron sulphate, and 0.25 gm. of ammonium sulphate. To some of this medium he added monoses, such as glucose, levulose, fructose and galactose. To some of it he added bioses, such as maltose, lactose and saccharose; and to some he added two polyoses, namely, dextrin and glycogen. In these he planted the thrush organisms. He found mycelium in the bioses and polyoses, but obtained no mycelium in the monoses.

He also believed that aerotropism brings about the mycelial forms. He said that all his stab cultures showed a characteristic growth at the top of the tube; where the oxygen tension was high he obtained only the yeast form; where the tension was low, namely, toward the bottom of the tube, he obtained the mycelial form.

He further illustrated this theory by the following experiment: He dissolved a tiny piece of sugar in several c.c. of saliva. To this he added a small amount of the yeast-like cells from a fresh malt culture. He then took a drop of this emulsion and placed it on a slide. The slide was covered with a cover glass and placed in a dish surrounded with wet filter paper so as to provide sufficient moisture. This was then placed in the incubator. At the end of twelve hours the preparation showed mycelial forms toward the center of the drop, for here the oxygen tension was low, and yeast-like forms at the edges.

Concerning the unity or plurality of species there is considerable difference of opinion. Stumpf¹⁴ declared that the filamentous and the globular forms were two different species of fungi. However, no other observer has agreed with this view. Fischer and Brebeck⁶ claimed that there were two varieties: one, a large-spored variety that liquefied beer wort gelatin and formed a pellicle on milk and wort, and formed endospores; the other, a small-spore

kind that does not liquefy gelatin nor does it form pellicles or endospores. Daireuva¹¹ has come to the conclusion that "neither the microscopic, pathologic or cultural aspects warrant the establishment of specific differences."

Sugars have been found useful in classifying bacteria, such as the coli typhoid group. No attempt that we know of has been made to classify the thrush organisms by this means. The authors who have mentioned sugars are Cao,¹⁵ Fisher and Brebeck,⁷ Daireuva,¹¹ Troisiér and Achalme,²⁰ and Denecke, quoted by Plaut.² Cao, in describing the thrush organism, best known in literature as *Oidium albicans*, stated that it attacked no sugars. Fischer and Brebeck said that the liquefying variety fermented dextrose, levulose and maltose but not saccharose. Daireuva stated that the parasite fermented glucose, levulose and maltose; that it consumed dextrin, mannite and glycerol without fermenting; that it did not utilize or ferment lactose, and utilized saccharose without inverting or fermenting. Denecke claimed that it fermented levulose, maltose, lactose but not saccharose. Troisiér and Achalme described a case clinically diagnosed as thrush. They did not, however, believe the organism isolated from the case to be the thrush parasite, but rather a yeast. One of the points on which they based this decision was the fact that their organism fermented saccharose and showed strong alcoholic fermentation. They believed that the thrush parasite did not do this.

Agglutination is another means in use in modern bacteriology for establishing species. Noisette, quoted by Plaut,² has carried out some agglutination tests with his thrush cultures. He says that on immunizing an animal with a thrush strain, the serum of that animal develops agglutinins, which agglutinate the specific strain that has been used for immunizing. He has tried this serum on various strains but has found that the serums will agglutinate only their own specific antigens. He has, therefore, concluded that there is not merely a single *Saccharomyces albicans*, but an entire class, which contains varieties.

Roger¹⁷ succeeded in obtaining agglutinins in immunized rabbits. More recently Widal and others,¹⁸ in studying agglutination with the serum of sporotrichosis cases, have discovered that the serum of patients who suffered from thrush will agglutinate the "conidia" of *Oidium albicans*, but only in low dilutions, 1:10 to 1:50, and that the same serum agglutinates more markedly *Sporotrichum* spores in dilutions of 1:40 to 1:50. This reaction with the spores of *Sporotrichum beurmanni* is so constant that they were able to use it in the diagnosis of thrush. With the exception of Noisette, no author has tried to establish the unity or plurality of species by means of agglutination.

I began the study of the thrush parasite with 2 strains on hand, T 2 and T 9; the first from the Army Medical School and the other from the university clinic, both from clinically diagnosed thrush cases. Within 6 months there were added 15 more strains; T 26 and T 27, from vaginal cases in pregnant women; T 13, from a case of conjunctivitis; T 11, T 12 and T M, from typical cases of mouth thrush; T L, from an ulcer in the mouth, and the remainder, T 14, T 16, T 18,

¹⁵ Ztschr. f. Hyg. und Infektionskr., 1900, 34, p. 282.

¹⁶ Arch. de méd. expér. et d'anal. path., 1893, 5, p. 29.

¹⁷ Compt. rend. Soc. de Biol., 1896, 48, p. 728.

¹⁸ Ann. d. l'Inst. Pasteur, 1910, 24, p. 1.

T 21, T 22, T 23, T 24, T 25, were obtained from throat cultures sent to the laboratory of the Minnesota State Board of Health. The latter were cultures taken for diagnosis of diphtheria. For purposes of comparison yeast strains were used. Of these Y 1 was a cultivated yeast, Y 2, Y 3, Y 4 and Y 5 were wild yeasts and Y G, Y 17 and Y 19 were yeasts isolated from the throat cultures sent to the laboratory of the State board of Health. A monilia, from a case of sprue, obtained from the Army Medical School, was also used.

Pure cultures of these strains were obtained by plating them on dextrose-tartaric acid agar. This medium is made by adding to 10 c c of melted agar 1 c c of a sterile dextrose tartaric acid solution (prepared by adding 50% of dextrose and 5% of tartaric acid to water, sterilized in the autoclave). The addition of the dextrose tartaric solution inhibits the growth of bacteria and makes easy the obtaining of pure cultures. A good growth is obtained within 2 days.

The surface colonies are round, wax-like, creamy, elevated and granular; while the deep colonies are irregularly surrounded with radiating mycelium. Some of these deep colonies are round and are surrounded with a fine branching mycelium so that they present a stellate appearance; others are torpedo shaped with the mycelium extending from one side.

On routine mediums my strains of the thrush organism gave no characteristic growth. They grew at the bottom of broth in flocculent form. On milk there was no change, nor did any pellicle form. On gelatin there was a smooth white growth on top. None of my strains liquefied maltose gelatin. On agar, the growth was fine and whitish; on potato, it was gray. The organism grows most easily and abundantly on Sabouraud agar. I also planted my strains, as well as some yeasts, on carrots. Both gave a snow white growth. This, therefore, cannot be used for the differentiation of the thrush organism as Liossier and Roux³ claim. The morphology of the organism has been described in the earlier part of this paper. As endospores were not observed in the numerous strains and wet preparations which I made from various mediums, I tried the gypsum block method, hoping to obtain them that way. Plaster of Paris was hardened, slanted, fashioned to fit into glass tubes, moistened with peptone solution or distilled water and autoclaved. The various strains of the parasite were inoculated on the slanted surface of the gypsum. No nutritive material was placed in the tubes, for I wanted to create unfavorable conditions which would result in sporulation, just as is the case with

yeasts. The tubes were kept at a temperature of 20 degrees for several days.

At the end of that time it could be seen that the cells became oval, larger and swollen. They became free from granules, the dancing figures were enlarged, and the vacuoles distended so that they looked like spores. Carefully made spore stains, using Moeller's method, showed none. The vacuoles appear so much like spores that one can easily be misled into believing them to be such.

An attempt was also made to verify the observation of chlamydospores so carefully and elaborately described by Linossier and Roux, using their medium. In this medium, tubed and sterilized, I planted my strains. I found chlamydospores in 2 of them, namely T L and T 2. Their appearance corresponds to the description given by Linossier and Roux. They were spherical and enclosed by a heavy membrane. Within could be seen the highly refractile globule surrounded by a corona of tiny granules. The chlamydospores were at the extremities of short hyphae. I was unable to squeeze out the globules or observe the germination of the chlamydospores.

The complete absence of ascospores or, in fact, of any sort of endospores in all of the strains studied, no matter what the medium, leads me to believe that such structures are not formed by the thrush parasite and that it should, therefore, be retained in the genus *Oidium* rather than the genus *Endomyces*.

Chlamydospores are not typical for any genus. They are common to many fungi, hence the finding of them in two of the strains does not affect the classification. I believe them to be a resistant form of spore arising when conditions become unfavorable.

In my cultures I found, as a rule, yeast-like cells on the surface of the solid mediums. However, there were exceptions. Some of the old agar slant cultures developed deep radiating mycelium. Certain of my dextrin and dextrose agar slants, on being subjected to anaerobiosis (described later), showed mycelium, as did also deep colonies in dextrose-tartaric acid agar.

Liquid mediums, as a rule, showed the mycelial form. Here again were exceptions. I have found only yeasts in Linossier's dextrose liquid medium and in another medium devised by Koser and Rettger¹⁹ and containing: water, 1,000 c c; sodium chloride, 4 gm.; KCl, 1 gm.; MgSO₄, 0.2 gm.; CaCl₂, 0.05 gm.; KH₂PO₄, 1 gm.; (NH₄)₂PO₄ 1 gm., and glycerol, 30 gm.

¹⁹ Jour. Infect. Dis., 1919, 24, p. 301.

The addition of carbohydrates to liquid mediums showed marked results. Linossier's medium was used, to which were added dextrose, galactose, or dextrin. These were made up in liquid form, or solidified with agar. The solid mediums gave only the yeast-like cells. Of the liquid mediums, dextrin and galactose gave profuse mycelium and dextrose gave only the globular form. Galactose is of about the same molecular weight as dextrose and, therefore, in accordance with Linossier's theory, should give the yeast cells only. I have also obtained mycelium in sugar-free medium, such as plain broth and peptone. These results seem to show that sugars have some influence on the formation of mycelium, but not to the extent to which Linossier and Roux would have us believe.

Two other experiments which I have carried out seem to indicate that other factors, besides the composition of the medium, influence the morphology of the organism. These experiments were carried out to observe the influence of oxygen and the surface tension of the medium.

Linossier's mineral liquid medium was made up with dextrose, dextrin and galactose, and is dextrin and dextrose agar slants. On these were planted 5 strains, namely, T 9, T 12, T 26, T M and T 16. The tubes were immediately placed in jars which were connected by means of glass tubing to a flask containing calcium carbonate. Just before adding hydrochloric acid to the calcium carbonate for the purpose of generating carbon dioxide, there was added to each jar some pyrogallic acid and sodium hydroxide solution. Carbon dioxide was then passed through the jars. When a match would no longer burn in the gas escaping from the last jar in the series, the apparatus was disconnected and the glass tubing quickly sealed. The jars, together with some controls grown aerobically, were placed in the incubator. After two days the cultures were examined. Table C shows the results. It shows that anaerobiosis brought about a mycelial growth on the solid medium of most of the strains planted, but there are some exceptions.

The work of Larson, Cantwell and Hartzell²⁰ on the effect of surface tension of the medium on the growth of bacteria has given interesting results. It was thought that the surface tension of the medium might have some influence on the growth of the thrush parasite, so the following experiment was made. Linossier's liquid medium

²⁰ Jour. Infect. Dis., 1919, 25, p. 41.

was again used. The surface tension was depressed by the addition of castor oil soap prepared as described by the authors. The dextrin solution without the soap had a surface tension of 50 dynes. The addition of 1% of a 2% solution of the castor oil soap depressed the tension to 44 dynes. The galactose medium had a tension of 48 dynes and 43 when depressed as above. Dextrose changed from 58 to 43 dynes. Seven strains, T 9, T 12, T 16, T 26, T M, T 2, and T L were

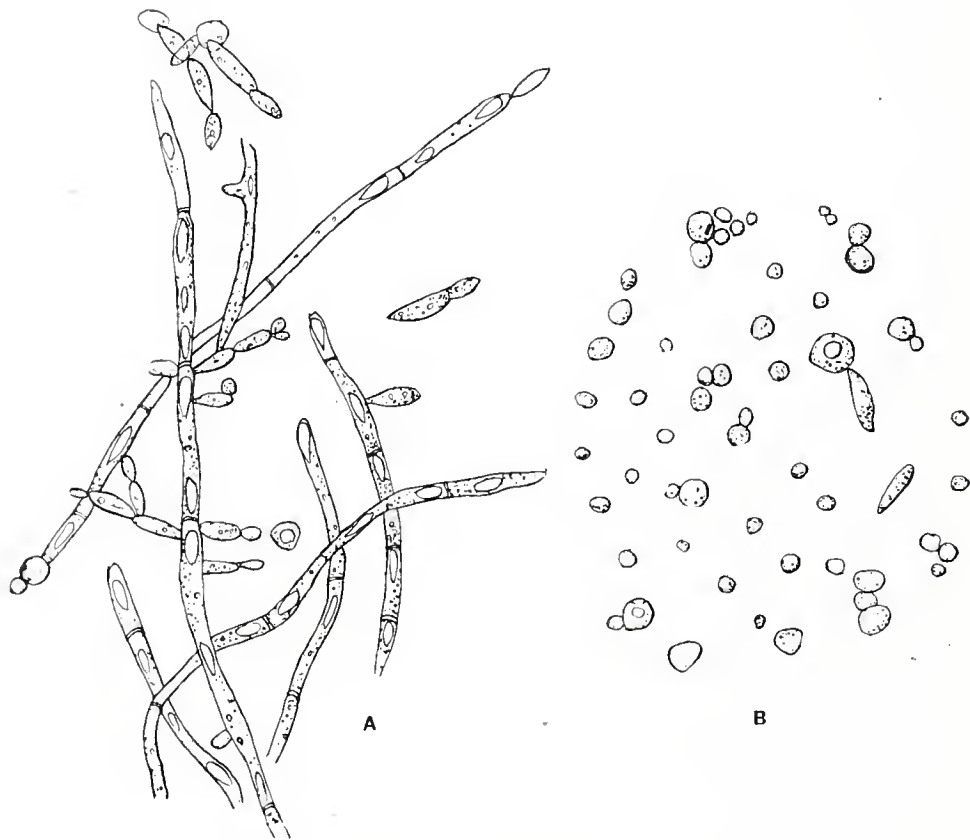


Fig. 1.—A, the morphology of *Oidium albicans* in Linossier's solution with dextrin, normal surface tension; B, the same strain grown in Linossier's solution with dextrin, the surface tension being depressed by castor oil soap.

inoculated simultaneously into tubes of these mediums, both of normal tension and of low tension as described, and incubated at 37 degrees. Table C shows the results after two days' growth. It shows that in the case of dextrin the filamentous form was present in controls, while yeast-like forms alone appeared when the tension was depressed. The results were most striking and definite. They are presented in figure 1, A.

With galactose, however, no such differentiation was observed, mycelium appearing in fluid of low tension as well as in the normal control tubes, while with dextrose also the addition of soap was without influence, the yeast form alone appearing in both series of tubes. After making these microscopic observations, the tubes of each of the inoculated mediums, both those of the normal tension and those of low tension, were centrifuged and the surface tension of the clear supernatant fluid was read. The surface tensions were determined by Mr. Green using Du Nouy's apparatus. The results are shown in the following table:

TABLE 1
SURFACE TENSION OF CLEAR SUPERNATANT FLUID AFTER CENTRIFUGATION OF MEDIUMS

	Uninoculated Normal Medium	Uninoculated Depressed Medium	Inoculated Normal Medium	Inoculated Depressed Medium
Dextrin.....	50 dynes	44 dynes	54.5 dynes	48 dynes
Galactose.....	48 dynes	43 dynes	65 dynes	49.5 dynes
Dextrose.....	53 dynes	43 dynes	58 dynes	46.5 dynes

It will be seen that the growth of the organism in every case raised the surface tension of the medium. This increase, however, is much more marked in the case of galactose and dextrose than dextrin, and is probably to be explained by the acid produced from the simpler sugars, which will precipitate soap.

Unfortunately these studies could not be carried further. It is quite clear, however, from the work done, that a multiplicity of factors determine the form which the thrush parasite may assume. In general, I may state that the yeast or unicellular form occurs in the optimum mediums. The organism grows most rapidly when the medium contains an abundance of the simpler fermentable carbohydrate. It is aerophilic and produces a more luxuriant growth on the surface of solid medium than in the depths of liquid medium. The unicellular form, the cells being spherical or oval, offers a smaller surface in proportion to the volume of the protoplasm than the cylindrical mycelium, but also affords the most rapid means of reproduction and dissemination of the organism.

It would seem that the yeast-like form is, therefore, the optimum form and that the mycelial form is only assumed in mediums poor in

oxygen, or in readily assimilable carbohydrates, this form possibly being better adapted by virtue of its larger surface for absorption and respiration.

The task of differentiating species of bacteria has been greatly aided by the use of sugars and agglutination tests. I resorted to these means in an attempt to determine the unity or plurality of species of thrush organisms.

TABLE 3
SUGAR FERMENTATIONS, SECOND SERIES

Strain	Dextrose		Galactose		Lactose		Levulose		Maltose		Mannite		Raffinose		Saccharose		Presence of Mycelium
	A	G	A	G	A	G	A	G	A	G	A	G	A	G	A	G	
YG.....	+	—	+	—	+	—	+	—	—	—	—	—	—	—	—	—	—
Y17.....	+	+	+	+	—	—	+	+	+	+	—	—	+	—	+	+	—
Y19.....	+	—	+	—	—	—	+	—	+	?	—	—	—	—	—	—	—
T2.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T9.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T11.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T12.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T13.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T14.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T16.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T18.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T21.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T22.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T23.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T24.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T25.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T26.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
T27.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
TL.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
TM.....	+	+	+	—	—	—	+	+	+	+	—	—	—	—	+	—	+
Monilia from sprue.....	+	+	+	+	—	—	+	+	+	+	—	—	+	—	+	+	+

"A" indicates acid, "G" indicates gas, "Y" indicates yeast strains, "T" indicates thrush strains.

Sugar broths were made by dissolving 1 gm. of the sugars to each 100 c c of beef extract broth, using Andrade's indicator. The following carbohydrates were used at first: dextrose, maltose, lactose, saccharose, mannite, glycerol, salicin, inulin, galactose, arabinose, levulose, amygdalin, xylose, erythrite, raffinose, dulcitol and starch. Into these were planted some strains of the thrush organism and some strains of yeasts as controls. Table 2 shows the results obtained. They seemed encouraging, for whereas the yeasts showed a variety of fermentations, the thrush strains presented uniform results. A fresh lot of medium was made. The list of sugars was limited to dextrose, galactose, levulose, maltose and saccharose since on the other sugars there was no action. Again the thrush strains fermented the

same sugars. Whenever I obtained a new strain of clinically diagnosed thrush I planted it in the sugars and invariably obtained the same results. All of the strains studied fermented the following sugars with the results indicated: dextrose acid and gas, galactose acid without gas, levulose acid and gas, maltose acid and gas, saccharose acid without gas.

On repetition of the experiment with all of the strains after several months' cultivation, the same results were obtained. From this experiment it is clear that the thrush parasite is constant in its sugar reactions and that these reactions do not indicate a plurality of species. All of the yeasts studied gave sugar reactions different from those of the thrush strains, so that the sugar reactions can be utilized for the identification of *Oidium albicans*. They are the more useful in that typical mycelium is formed readily in many of the sugar broths, most constantly and abundantly in galactose.

TABLE 4
INFLUENCE OF OXYGEN AND SURFACE TENSION ON MORPHOLOGY

	Aerobic					Anaerobic					Liquid					
	Solid		Liquid			Solid		Liquid			Normal Tension			Low Tension		
	Dex-trin	Dex-trose	Dex-trin	Ga-lac-tose	Dex-trose	Dex-trin	Dex-trose	Dex-trin	Ga-lac-tose	Dex-trose	Dex-trin	Ga-lac-tose	Dex-trose	Dex-trin	Ga-lac-tose	Dex-trose
T9	Y	Y	M	M	Y	M	Y	M	M	Y	M	M	Y	Y	M	Y
T12	Y	Y	M	M	Y	M	M	M	M	Y	M	M	Y	Y	M	Y
T26	Y	Y	M	M	Y	M	M	M	M	Y	M	M	Y	Y	M	Y
TM	Y	Y	M	M	Y	Y	M	M	M	Y	M	M	Y	Y	M	Y
T16	Y	Y	M	M	Y	M	M	M	M	Y						
T2	M	M	Y	Y	M	Y
T21	M	M	Y	Y	M	Y
TL	M	M	Y	Y	M	Y

"Y" indicates yeast cells, "M" indicates mycelium.

For my agglutination experiments antigens were made from several strains as follows: 10 c c of a sterile 0.8% salt solution with 0.25% of tricresol was added to each of the Sabouraud agar slants. The growth was gently emulsified. The emulsions were transferred to clean tubes. They were heated for one hour at 56° C. in the water bath. A little of each emulsion was planted on a fresh medium and inspected the next day for growth. If they showed growth, the emulsions were again heated and tested. Of such emulsions, 2 c c were injected intraperitoneally into rabbits 4 times, 3 days apart. One week after the last injection they were bled and the serums collected were

used for microscopic agglutination tests in dilutions of 1:10, 1:20 and 1:50. The emulsions used as antigens were prepared by scraping the growth from Sabouraud agar slants into salt solution. This gave emulsions which slowly sedimented but became uniformly turbid on slight agitation. After making the mixtures they were incubated at 37 C. for 2 hours and then placed in the icebox over night. In the first experiment the following strains were used for immunization: T 2, T 3, T 9, T 11 and T L. The same strains were used as antigens. Serum T L agglutinated the homologous strain and strain T 9, both in a dilution of 1:20. Serum T 9 agglutinated its homologous strain at a dilution of 1:50 and strain T L at 1:10. Otherwise no agglutination occurred.

After one more injection the serum of the rabbit inoculated with T 9 was used for a further experiment using, in addition to the yeast and thrush strains mentioned, 2 emulsions of spores of *Sporotrichum schenckii*. These were both isolated from cases of cutaneous sporotrichosis. A further test was made using the serums of the 2 cases of vaginal thrush with their homologous strains and with emulsions of sporotrichum spores. The results were completely negative.

It would appear from these experiments that the agglutinins are not formed in sufficient quantity either in experimentally inoculated animals or in clinical cases of thrush to be of diagnostic or differential value. I am unable to confirm with *Sporotrichum schenckii* the observation of Widal and others¹⁸ with *Sporotrichum beurmanni*.

SUMMARY AND CONCLUSIONS

Seventeen strains of the thrush parasite proved identical and constant in their morphologic and cultural characters. They all corresponded to the nonliquefying type of Fischer and Brebeck.

Carbohydrate mediums were fermented uniformly and constantly by all strains. They are of value in the identification of the species.

Agglutinins are not produced by the thrush parasite in sufficient quantity to be of diagnostic or differential value.

The thrush parasite produces chlamydospores but not ascospores. It is correctly placed in the genus *Oidium*.

The organism tends to assume a mycelial form in liquid mediums, in mediums containing complex carbohydrate, in mediums of low oxy-

gen tension, and in mediums of low surface tension, while the unicellular or yeast-like form occurs in solid mediums, in the presence of simple carbohydrates, an abundance of oxygen, or mediums of higher surface tension. These factors may be interrelated, while other factors as yet unknown may affect the morphology. It is suggested that pleomorphism of this organism is an attempt at adaptation, the mycelial form developing in relatively unfavorable conditions.

ACUTE RESPIRATORY INFECTION IN MAN FOLLOWING INOCULATION WITH VIRULENT BACILLUS INFLUENZAE

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The two epidemics of influenza which have swept over the country since the fall of 1918 have served to concentrate the attention of bacteriologists once more on the etiology of this disease, and a considerable amount of their interest has been centered on the part played by the influenza bacillus. During the last two years a number of investigators have tried to produce influenza in man by direct inoculation of *B. influenzae* cultures into the healthy nose and throat. Almost without exception, however, these efforts have resulted in failure.

Davis¹ succeeded in producing symptoms in a young man whose nose and throat he inoculated with a thick emulsion of influenza bacilli. Forty-eight hours after inoculation the patient complained of chilly sensations, headache and weakness; temperature 100.2, leukocytes 9,200. The throat was reddened and covered with mucus. The patient also developed a cough with mucopurulent expectoration. The acute symptoms lasted only 3 days. Cultures from nose, throat, and sputum showed many colonies of *B. influenzae*. The patient carried influenza bacilli in his throat for 4 weeks. The culture used by Davis was not isolated from a case of influenza, but from an uncomplicated case of pertussis.

Sellards and Sturm² inoculated human volunteers with a mixture of 5 strains of *B. influenzae* which had been isolated from cases of measles. These cultures had been under cultivation for 6-8 weeks before the tests were made. None of the volunteers developed any symptoms of measles or influenza, nor did they become carriers of influenza bacilli for any length of time.

Bloomfield³ observed the effect of inoculating the nose and throat of human volunteers with strains of influenza bacilli isolated from the throats of healthy men. The 3 strains employed had all been under artificial cultivation for several weeks at the time of the experiments. Altogether 14 volunteers were tested. Four received the inoculation on the tongue; 5 in the nose; 3 on the tonsils; and 2 in the nasopharynx. None of the volunteers showed any symptoms, and none of them became carriers. In Bloomfield's experiments influenza bacilli had usually disappeared in cultures taken from the nose and throat 24 hours after inoculation.

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¹ Jour. Am. Med. Assn., 1919, 72, p. 1317.

² Bull. Johns Hop. Hosp., 1919, 30, p. 331.

³ Bull. Johns Hop. Hosp., 1920, 31, p. 85.

A number of investigators have inoculated human volunteers with strains of *B. influenzae* isolated from cases of clinical influenza. Wahl, White and Lyall⁴ inoculated volunteers with cultures of *B. influenzae*, spraying the nose and throat with an emulsion washed from a plate of chocolate agar. First the authors used old cultures, but as no results were obtained, the experiment was repeated four days later on two of the same volunteers with a freshly isolated strain (second generation). Of the two volunteers who received the fresh culture, one had no symptoms, while the other experienced only a slight local reaction in the nose on the afternoon of the injection. In these experiments, influenza bacilli persisted in the throats of the volunteers for 1-3 weeks or even longer.

The United States Public Health Service in conjunction with the U. S. Navy Medical Department carried out some interesting experiments with the influenza bacillus just after the epidemic of 1918. In Boston, M. J. Rosenau and his co-workers⁵ injected a freshly isolated culture of *B. influenzae* into the nostrils of 6 volunteers, 3 of whom had a history of recent influenza and 3 of whom had no such history; none of the 6 developed symptoms. At the same time in San Francisco, McCoy and Richey⁶ inoculated 5 volunteers with a heavy suspension composed of 8 strains of *B. influenzae*. Although none of this latter group of volunteers had had influenza, they all failed to show any symptoms following the inoculation.

From this review of the literature, it will be observed that, in the first place, the strains of influenza bacillus employed by these various investigators had, with two exceptions, been isolated from healthy individuals or from cases of measles or whooping cough. In the second place, the cultures used had usually been subjected to artificial cultivation for weeks or even months before the inoculations were made. The rapidity with which the influenza bacillus loses its virulence is well known. Finally, it has been shown that strains of influenza bacilli, like those of pneumococci, streptococci and other bacteria, differ greatly in their virulence. Some strains are entirely avirulent.

Blake and Cecil⁷ recently have shown that by inoculating the nose and throat of monkeys with a virulent culture of *B. influenzae*, an acute infection of the upper respiratory tract may be induced, which is quite similar to influenza in man. In these experiments, the strain of influenza bacillus employed was first rendered virulent for the species by repeated intraperitoneal injections. By this procedure, the culture eventually became so virulent for monkeys that inoculation of the nose and throat with only a small quantity of either the peritoneal exudate or a blood broth culture would initiate an acute respiratory infection.

⁴ Jour. Infect. Dis., 1919, 25, p. 419.

⁵ U. S. Public Health Rep., 1919, 34, p. 33.

⁶ U. S. Public Health Rep., 1919, 34, p. 33.

⁷ Jour. Exper. Med., 1920, 32, p. 691.

characterized by sneezing, coughing, mucopurulent discharge, leukopenia, prostration and fever. Furthermore, several of the monkeys thus infected later developed a hemorrhagic bronchopneumonia from which *B. influenzae* was recovered at necropsy.

If monkeys could be infected so readily with a virulent strain of *B. influenzae*, it seemed reasonable to suppose that, by use of proper methods, a similar experimental disease could be produced in man. The experiments reported in this paper were undertaken with such an object in view.

METHODS

Culture Mediums.—The culture mediums consisted of freshly prepared "chocolate blood" agar or broth, and the sodium oleate hemoglobin agar of Avery. The chocolate medium was prepared by adding defibrinated horse or rabbit blood to veal-infusion agar or broth, and the mixture heated at 70 C. for a few moments until the blood changed color. The reaction of the medium was P_H 7.4 to 7.6.

Cultures.—Two strains of *B. influenzae* were employed in these experiments. Both were isolated in almost pure culture from patients with typical cases of influenza admitted to the Willard Parker Hospital during the epidemic of February, 1920. These two strains presented all the morphologic and cultural characteristics of the Pfeiffer bacillus and failed to grow on blood-free mediums.

Strain "Graham" was cultivated from the throat of a child with influenza and influenzal pneumonia. This strain was used in only one experiment. As the results were negative, it was discarded and another culture (strain "Wick") was substituted for the remaining experiments.

Strain "Wick" was isolated by Dr. A. W. Williams in conjunction with *Streptococcus hemolyticus* from the pleural exudate of a young woman who died of influenzal pneumonia and empyema. This strain was found to be virulent for both rabbits and mice. A rabbit injected intraperitoneally with 2 c c of a young chocolate blood broth culture died in 5 hours, and 0.25 c c of an 18-hour culture of the same strain killed a mouse in less than 24 hours.

Volunteers.—The volunteers selected for these experiments were healthy adults, either medical students or laboratory workers. Of the 6 persons inoculated with *B. influenzae* 3 gave a history of influenza during the epidemic of 1918-19. The other 3 denied having had the disease. Preliminary cultures were taken in every case from the nose

and throat to eliminate influenza bacillus carriers. One carrier, however, was purposely inoculated in order to determine whether the presence of influenza bacilli in the nose and throat conferred immunity against experimental infection. No volunteers were accepted who gave a history of any recent respiratory infection.

In addition to the 6 volunteers inoculated with influenza bacilli, 6 other volunteers were selected for control inoculations. Two of these received the filtrate from a chocolate blood broth culture of *B. influenzae*; 2 were inoculated with a virulent *Streptococcus hemolyticus* and the remaining 2 were tested with a group IV pneumococcus, which was highly virulent for mice.

All volunteers were kept under close observation during the period of the experiment. Temperature, leukocyte counts and cultures were frequently taken, and the subjects were carefully examined twice a day for any local or general reaction. When the influenza bacillus was recovered from cultures taken from the volunteers subsequent to inoculation, the strain was studied by means of agglutination and absorption tests to establish its identity with the strain inoculated. These tests were carried out by Dr. Olga R. Povitzsky of the Research Laboratory of the New York City Department of Health in connection with a biologic study of influenza bacilli undertaken by her simultaneously with the experiments herewith reported. Dr. Povitzsky's results are reported in detail in the *Journal of Immunology* for January, 1921.

INOCULATION OF VOLUNTEERS WITH WASHINGS FROM CHOCOLATE BLOOD-AGAR CULTURES OF *BACILLUS INFLUENZAE*

The nose and throat of two healthy volunteers were inoculated with freshly isolated strains of *B. influenzae*, which had been cultivated for 18 hours on chocolate blood agar slants. The growth was washed from each slant with 1 c.c. of sterile broth, and made a fairly thick suspension. One of these volunteers, (case 1) gave a history of influenza during the epidemic of 1918; the other (case 2) denied having ever had the disease. Preliminary cultures showed that neither of these volunteers carried influenza bacilli.

CASE 1.—L. B., a woman aged 28, had never had pneumonia; influenza in Oct., 1918. Rarely had colds. Last cold in Nov., 1919. Had never received influenza bacillus vaccine. No recent exposure to influenza. Well nourished; blond; throat and tonsils normal; slight deviation of nasal septum to right.

Feb. 14, 1920: Preliminary cultures from nose and throat on sodium oleate-agar plates show colonies of streptococci, staphylococci and diphtheroid bacilli; no influenza bacilli. Feb. 16, 11 a. m.: Received in each nostril one-half slant, in 0.5 c c of broth, of an 18-hour chocolate blood-agar culture of *B. influenzae*, strain "Graham." The culture used was the second generation on artificial medium. Most of the suspension trickled back into the nasopharynx and was spit out. At 2 p. m.: Complained of frontal headache; 5 p. m., headache severe. No reaction in nose or throat. Temperature normal. Right Nostril: Smears showed mucus, a few pus cells and intracellular influenza bacilli. Cultures showed staphylococcus albus and diphtheroid bacillus; no influenza bacilli. Left Nostril: smears, no pus cells, no influenza bacilli; cultures same as right nostril. Throat smears negative. Cultures: staphylococcus albus, streptococcus and a gram-negative diplococcus. No colonies of *B. influenzae*.

Feb. 17, 10:30 a. m.: Had a comfortable night. Awoke in the morning feeling well except for a slight rawness in the throat and some obstruction in the nose. 5 p. m.: She had noticed slight malaise all day.

Feb. 18: Malaise continued; no other symptoms.

Feb. 19: She felt slight malaise.

Feb. 20: She felt well.

In this case one-half slant of influenza bacillus culture produced practically no local symptoms. There was slight malaise for several days following the inoculation and a frontal headache, which lasted only 24 hours. Influenza bacilli disappeared from the nose and throat in a remarkably short time. Cultures taken 6 hours after inoculation were entirely free from influenza bacilli.

The next volunteer, case 2, received an entire chocolate-agar slant of *B. influenzae* culture in each nostril. A different strain (Strain "Wick") was employed for the inoculation (table 1).

CASE 2.—C. A. S., a man, aged 24, had never had influenza, though frequently exposed during the epidemic of 1918. No history of pneumonia. He had never received influenza vaccine. Last cold in Dec., 1919. He had no respiratory infection and had not been exposed recently to influenza. Physical examination revealed a well developed young man; his nose and throat were normal.

Feb. 10, 1920: Preliminary cultures from the nose and throat on blood-agar and chocolate blood-agar plates showed a predominance of *Staphylococcus aureus* and albus. Numerous colonies of *Streptococcus viridans* and a few colonies of a large gram-negative bacillus; no colonies of *B. influenzae*.

Feb. 12, 11 a. m.: The patient received, in each nostril, one entire slant (in 1 c c of broth) of an 18-hour chocolate blood-agar culture of *B. influenzae*, strain "Wick." The culture used was the third generation on artificial culture. A small quantity of the fluid passed back into the nasopharynx. 1:30 p. m.: The patient complained of headache and a burning sensation in the nose, accompanied by considerable serous discharge from both nostrils. Smears from nasal cavities taken at this time showed many influenza-like

TABLE 1
INOCULATIONS WITH CULTURES AND FILTRATES OF B. INFLUENZAE AND WITH
STREPTOCOCCUS AND PNEUMOCOCCUS CULTURES

Volunteer	Age	History of Influenza	Dose in Each Nostril	Symptoms	Duration of Symptoms	Microscopic and Bacteriologic Results
1. M. B.	28	Fall of 1918	One half slant of culture of influenza bacillus	Headache, rawness throat, nasal obstruction, malaise	3 days	Smears: mucus and a few pus cells. Cultures: no influenza bacilli 6 hours after inoculation
2. C. A. S.	24	None	One slant of influenza bacillus	Headache, rhinitis, pharyngitis, malaise	2 days	Smears: many pus cells and influenza bacilli. Cultures: positive after 8 hrs., negative after 24 hrs.
3. E. M. C.	28	Fall of 1918	0.1 c c exudate	Headache, rhinitis, malaise, pharyngitis, tracheitis, backache, soreness in chest, diplopia	10 days	Many pus cells, many influenza bacilli; bacilli in cultures 2 weeks after inoculation
4. F. J. L.	47	Fall of 1918	0.1 c c exudate	Rhinitis, pharyngitis, conjunctivitis, headache, malaise, general muscular aching	3 days	Mucus, pus cells, a few influenza bacilli which were present in cultures 10 days after inoculation
5. W. J. S.	39	None	0.5 c c 6-hour chocolate brown culture of influenza bacillus	Headache, rhinitis, pharyngitis, conjunctivitis, tracheitis, backache, pain in legs	4 days	Many pus cells, many influenza bacilli in smears and cultures 5 days after inoculation
6. T. B.	32	None Influenza bacillus carrier	0.5 c c 6-hour chocolate brown culture of influenza bacillus	Rhinitis, malaise, headache, pharyngitis, tracheitis, soreness in chest	3 days	Many pus cells, many influenza bacilli in smears and cultures after inoculation
7. F. K.	21	None	0.5 c c filtrate 6-hour chocolate-broth culture of influenza bacillus	None		
8. M. K.	25	None	0.5 c c filtrate 6-hour chocolate-broth culture of influenza bacillus	None		
9. R. S.	21	None	0.5 c c 6-hour culture of streptococcus hemolyticus	Slight dryness and redness of throat	A few hours	Streptococcus hemolyticus in cultures 6 days after inoculation
10. H. W.	20	None	0.5 c c 6-hour culture of streptococcus hemolyticus	Headache, fever, leukocytosis, sore throat, exudate on tonsils	3 days	Streptococcus hemolyticus in cultures 4 days after inoculation
11. H. Z.	20	None	0.5 c c 6-hour culture of pneumococcus 4	None	Pneumococcus 4 in cultures 48 hours after inoculation
12. C. W.	29	None	0.5 c c 6-hour culture of pneumococcus 4	Slight dryness in throat on third day	Pneumococcus 4 in cultures 4 days after inoculation

bacilli. 3:30 p. m.: He complained of general malaise and drowsiness. 6:30 p. m.: The drowsiness was marked. He complained of rawness in throat and tender glands at angles of the jaw. There was a small amount of blood in the serous discharge from the left nostril and marked anorexia. The patient looked pale and sick; the nasal mucous membrane was swollen and reddened, and there was considerable serous discharge; the throat was intensely reddened and congested. The temperature was 97; pulse, 96. Smears from nasal cavities showed many pus cells and a few influenza-like bacilli, mostly intracellular. Cultures from the throat contained a few colonies of *B. influenzae* and a few colonies of a diphtheroid bacillus; from nasal cavities, no colonies of *B. influenzae*.

Feb. 13, 11 a. m.: Patient complained of headache and dizziness. There was complete obstruction of both nasal cavities early in the morning and considerable obstruction still existed. The patient had developed a dry, hacking cough. He woke up once during the night with burning and watering of the eyes. The nasal discharge was still abundant and the patient had to blow his nose frequently. Anorexia and general malaise persisted. The pallor was quite noticeable. The patient presented the picture of a mild influenzal attack; the mucous membrane of nose was still red and swollen; throat congested and covered with mucus. Smears from right nasal cavity showed a moderate number of pus cells but no influenza bacilli. Smears from the left nasal cavity showed many pus cells and a few influenza bacilli, both intracellular and extracellular. Cultures right nasal cavity, showed no colonies of *B. influenzae*; a few colonies of *staphylococcus albus*; from left nasal cavity no colonies of *B. influenzae*. There were a few colonies of a gram-negative diplococcus and a few colonies of a diphtheroid bacillus. Cultures from the throat showed many gram-negative diplococci and staphylococci. There were no *B. influenzae* colonies on the plate. 4 p. m.: Patient complained of feeling very tired. Temperature, 98 degrees. Sent home to bed.

Feb. 14, 10:30 a. m.: Patient felt better. Moderate amount of secretion still present in nasal cavity. Smears from nasal cavity showed mucus, desquamated epithelium and a few pus cells; no influenza bacilli.

Feb. 15: Patient felt well.

This volunteer appears to have been definitely infected by the inoculation. The local and constitutional symptoms were quite definite, and influenza bacilli were present in smears from the nostrils 24 hours after the injection of the culture. The temperature and leukocytes were unchanged. The most striking features were headache and general malaise, and the well marked local inflammation in the nose and throat.

From these two experiments it would appear that an acute but rather mild respiratory infection may be produced by the inoculation of chocolate blood-agar slant cultures of *B. influenzae*, if a sufficiently large dose of culture is employed. In view of the rapid disappearance of influenza bacilli in these two cases, the possibility of the reactions being of a toxic, rather than an infectious, nature cannot be excluded.

INOCULATION OF VOLUNTEERS WITH THE PERITONEAL EXUDATE FROM
A MONKEY WITH *BACILLUS INFLUENZAE* PERITONITIS

Blake and Cecil⁷ were successful in producing an acute respiratory disease in monkeys by direct inoculation with the peritoneal exudate from a monkey with *B. influenzae* peritonitis. It seemed desirable, therefore, to try this method of experimental infection on human volunteers. The exudate was obtained by inoculating a rhesus monkey intraperitoneally with a large amount (20 plates) of influenza bacillus culture. Twenty-four hours later the monkey was dead. The cloudy exudate, containing large numbers of influenza bacilli in pure culture, was removed from the peritoneal cavity with a sterile pipet and was injected immediately into the nostrils of the volunteers.

Two volunteers were inoculated with peritoneal exudate. The dose in both cases was quite small, only 0.1 cc of exudate in each nostril. In addition, the throats of the volunteers were rubbed with a cotton swab which had been soaked in the exudate.

CASE 3.—E. M. C., a man, aged 28, not susceptible to respiratory infections, had never had pneumonia. He had had a mild attack of influenza in the fall of 1918. He had had a mild cold in Oct., 1919. Free from cold and cough at present. Physical examination showed a well developed young man. Nasal passages clear. Mild chronic pharyngitis.

April 12, 1920: Preliminary cultures from nasal cavities showed staphylococcus albus. No colonies of *B. influenzae* on any of the plates.

April 13, 10 a. m.: Received 0.1 cc of *B. influenzae* exudate (strain "Wick") in each nostril, and throat was swabbed with *B. influenzae* exudate. 4 p. m.: Patient complained of slight headache and was sneezing. No malaise. Cultures from nasal cavities showed a few colonies of *B. influenzae*, and a few colonies of staphylococcus albus. The *B. influenzae* strain was biologically identical with strain "Wick." Culture from throat showed chiefly streptococcus viridans.

April 14, 10 a. m.: Comfortable night. In the morning there were no constitutional symptoms, but the patient complained of stuffiness in the nose. Smears from nose and throat were negative. Cultures from left nostril showed a few colonies of *B. influenzae* and staphylococcus albus. Cultures from right nostril showed a predominance of *B. influenzae*. Throat: streptococcus, Staphylococcus albus and *B. influenzae*.

April 15, 10 a. m.: General malaise; patient felt lazy and tired. Obstruction in nasal cavities and watery discharge from nose, particularly the right side. Cultures from right nostril showed almost pure growth of *B. influenzae*; from the left nostril, a few colonies of *B. influenzae* and Staphylococcus albus. Throat: Moderate number of *B. influenzae* colonies together with usual mouth flora. The *B. influenzae* strain isolated was biologically identical with Strain "Wick."

April 16, 10 a. m.: Patient felt better, except for cold in the head. Nasal cavities were still stopped up. Cultures from nose and throat positive for *B. influenzae*.

April 19: Still had mild cold in head. Cultures from throat and right nostril were positive for *B. influenzae*.

April 20, 10 a. m.: Cold in head persisted. Yesterday patient had long automobile ride. Wore no overcoat. Today complained of a recurrence of general malaise.

April 21: Patient was sneezing and coughing frequently, with scanty expectoration; no pain or discomfort, but general malaise was marked. Temperature 98 F. Went to bed. Smears from right nostril showed pus and influenza bacilli. Cultures: Almost pure growth of *B. influenzae*; smears from left nostril showed pus and many influenza bacilli; cultures, same as on right. Cultures from throat, positive for influenza bacilli. Smears from sputum showed mucus and pus; no bacteria. Cultures showed a few colonies of *B. influenzae*. The *B. influenzae* strain isolated was biologically different from Strain "Wick."

April 22: In bed most of day. Felt weak and inert. Profuse watery secretion from nose; cough, with mucopurulent expectoration. Soreness of muscles of chest; severe frontal headache and backache; diplopia and heaviness of eyelids. Temperature 99.4 F. Smears from nasal cavities showed pus and influenza bacilli. Cultures: almost pure growth of *B. influenzae*. Cultures from throat positive for *B. influenzae*. Smears from sputum showed many pus cells and many influenza bacilli. Cultures from sputum showed streptococci, staphylococci and influenza bacilli (about 20% of colonies).

April 23: Had severe coughing spell during night. Profuse nasal discharge continued; headache not so severe. The patient was pale and looked sick; dark rings about the eyes. Nasal mucous membrane was congested. Nasal discharge sometimes contained blood. His throat was still markedly inflamed. Cultures from nasal cavities showed scanty growths; no *B. influenzae* colonies. Cultures from throat also showed a large number of colonies of *Streptococcus hemolyticus*.

April 24: Patient looked and felt much better. Still had cold in head and coughed occasionally. Slight stiffness in back persisted.

April 25: Condition practically normal.

April 27: Felt all right. Cultures from throat showed no colonies of *B. influenzae*.

May 5: Patient continued well. Cultures from throat showed no colonies of *B. influenzae*.

This case was particularly interesting on account of the relapse which the patient experienced one week following the inoculation. The mild infection which developed immediately after the inoculation of *B. influenzae* had about run its course when, following exposure to cold, the patient developed a much more severe respiratory infection which involved the nose and throat and trachea and which necessitated his going to bed. Agglutination and absorption tests carried out with the cultures of *B. influenzae* isolated during the relapse indicated that this strain was entirely distinct biologically from the strain which was originally inoculated, and which had been isolated from the nose and throat during the first mild infection. The temperature and leukocytes in this case showed practically no deviation from normal. There was

a slight elevation of temperature at the onset of the relapse, but this was only temporary. It should also be noted that during the relapse the hemolytic streptococcus made its appearance in the cultures from the throat.

The next volunteer, case 4, received the same dose of peritoneal exudate that the volunteer in case 3 received. In spite of the small quantity of fluid injected (0.1 c c in each nostril), he promptly developed a characteristic chain of symptoms, as the following protocol will show (table 1, text-fig. 1).

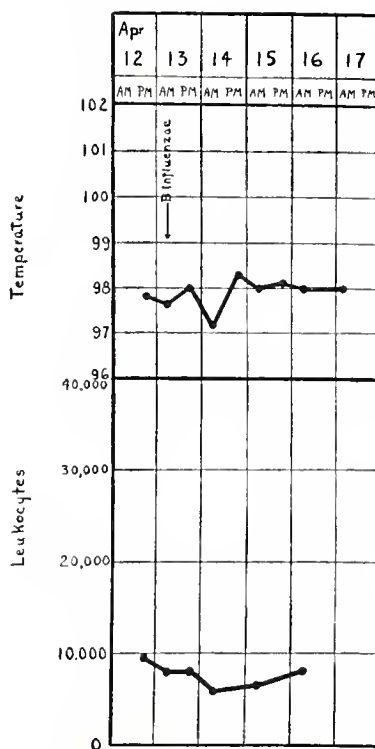


Fig. 1.—Case 4, F. L.; 0.1 c c *B. influenzae* exudate in each nostril.

CASE 4.—F. J. L., a man, aged 47, a laboratory technician, had influenza during the epidemic of 1918. There was no history of pneumonia; he rarely had a cold. He had had no colds at all during this fall and winter. A small swarthy man, well developed and well nourished. Pharynx and nasal passages normal.

April 12, 1920: Preliminary cultures from nostrils showed a few colonies of *Staphylococcus albus* and an occasional colony of a gram-negative diplococcus; no influenza bacilli. Cultures from throat; *Streptococcus viridans*, *Staphylococcus albus* and a gram-positive bacillus; no colonies of *B. influenzae*.

April 13, 11 a. m.: Received in each nostril, 0.1 c c of peritoneal exudate from a monkey with *B. influenzae* peritonitis (strain "Wick"). Throat rubbed with a cotton swab soaked in exudate. 1:30 p. m.: Nose began to run;

sneezed every few minutes; eyes were watering; throat slightly sore and raw. 4 p. m.: Patient complained of drowsiness and headache. Profuse serous discharge from nose. Nasal and pharyngeal mucous membrane swollen and reddened. Temperature 98 F. Cultures from left nostril were almost pure culture of *B. influenzae*. Throat culture showed a variety of bacteria; a few colonies of *B. influenzae*. Ten colonies of *B. influenzae* picked at random from plates were biologically identical with strain of *B. influenzae* inoculated.

April 14, 10 a. m.: Uncomfortable night, referable to headache, sore throat and copious nasal discharge. In the morning nose was stopped up and eyes were watering. He was still sneezing, but not so frequently. Complained of frontal headache and marked malaise. Patient presented the picture of a severe coryza. Mild conjunctivitis; mucous membrane of nose and throat reddened. Temperature, 97.2 F. Smears from the nasal cavities showed mucus, pus and a few influenza bacilli. Cultures from the nasal cavities gave almost pure growth of *B. influenzae* on both sides. The strain was biologically identical with strain "Wick." Cultures from throat yielded *Streptococcus viridans*, *Staphylococcus albus*, large gram-negative bacillus and a few colonies of *B. influenzae*. 4 p. m.: He went to bed on account of headache, dizziness and general malaise; complained also of pain in extremities, especially in elbows. Profuse diaphoresis; felt feverish. His temperature, however, was only 98 F.

April 15, 10 a. m.: Felt better, but malaise was still quite marked. Remained in bed a considerable part of the day. Not so much soreness in extremities, and headache was less severe. Nose was still discharging and both nasal cavities were obstructed. Cultures from right nostril showed *Staphylococcus albus*; left nostril, *Staphylococcus albus* and one colony of *B. influenzae*. Throat, usual mouth flora, and a few colonies of *B. influenzae*. Cultures from the conjunctiva showed *Staphylococcus albus* but no influenza bacilli.

April 16, 10 a. m.: Felt much better; returned to work. Nasal discharge much diminished. Smear from right nostril showed moderate amount of pus, no influenza bacilli. Cultures from right nostril showed *Staphylococcus albus* and a gram-positive bacillus; no influenza bacilli. Smears from left nostril showed pus cells and a few influenza bacilli. Cultures from left nostril showed no influenza bacilli. About 40% of the colonies in cultures from the throat were those of *B. influenzae*. Strain was biologically identical with "Wick" strain.

April 17: Condition was almost normal.

April 19: Patient felt all right. Cultures from right nostril were negative. Culture from left nostril showed 4 colonies of *B. influenzae*.

April 23: Culture from throat showed that 50% of colonies were *B. influenzae*. Strain was still biologically identical with "Wick" strain. Cultures from nostrils were negative.

May 5: Culture from throat showed 40% of *B. influenzae* colonies; strain identical with "Wick" strain.

This patient presented the picture of a mild, afebrile case of influenza. The prostration was quite marked and the headache and pains in the extremities were also characteristic. The leukocytes showed a definite leukopenia, the count going down from 9,300 to 6,000 (Fig.1). The temperature, as in the previous cases, remained normal; it was sub-

normal on the day following the inoculation. It is interesting to note that in this case influenza bacilli were still present in large numbers in the patient's throat four weeks after the inoculation.

INOCULATION OF VOLUNTEERS WITH YOUNG CHOCOLATE BLOOD-BROTH CULTURES OF *B. INFLUENZAE*

Two volunteers were inoculated with a 6-hour chocolate blood-broth culture of *B. influenzae*. The strain used for these inoculations was the same one used in the previous experiments with peritoneal exudate, but in this instance it was freshly isolated from the throat of the patient in Case 4. As in the two previous cases, very small quantities of culture were inoculated. These 2 volunteers each received 0.5 c.c. of culture in either nostril, but as the culture was very young, the actual number of bacteria injected was comparatively small. We were prompted to use the young cultures by the work of Parker,⁸ who found that 6-hour cultures of *B. influenzae* were more toxic than older cultures.

CASE 5.—W. J. S., a man, aged 39; had never had influenza though frequently exposed. No history of pneumonia. Not susceptible to colds; had had only two mild colds during the past 6 years; no recent colds. A fairly well nourished man but rather poorly developed physically. Nose and throat normal.

April 19: Preliminary cultures from nose and throat showed normal mouth flora. No colonies of *B. influenzae* on any of the plates.

April 20, 10 a. m.: Received 0.5 c.c. of a 6-hour chocolate blood-broth culture of *B. influenzae*, strain "Wick," in either nostril. In addition, the throat was rubbed with a cotton swab soaked in a culture of the same organism. Culture used was the third generation on artificial culture. 12:30 p. m.: Complained of sharp headache. 3 p. m.: Sneezed frequently. Nose felt stopped up and there was a watery discharge from the nostrils. Throat was getting sore, and there was some pain in the right ear. In addition to these local symptoms there was general malaise, drowsiness, and aching in the shoulders. Patient looked moderately sick. Mucous membrane of nose and throat reddened. Smears from right nostril showed a few pus cells and intracellular bacilli. Cultures showed streptococci but no colonies of *B. influenzae*. Smears from left nostril were similar to those of right nostril. Cultures from left nostril showed no influenza bacilli. Culture from throat showed a few colonies of *B. influenzae*. Strain was biologically identical with strain "Wick."

April 21, 10 a. m.: Patient had a very uncomfortable night. Suffered from extreme exhaustion, severe frontal headache and insomnia. Locally there was obstruction in both nostrils, watery discharge from the nose, sore throat, burning of the eyes, tenderness of the glands in the neck, soreness in the chest and profuse diaphoresis. In the morning many of the symptoms persisted, and in addition patient had developed a hacking cough. He also complained of backache and pain down right leg. Patient looked pale and sick; throat congested, profuse nasal discharge. 4 p. m.: Patient had severe headache and backache, pain in the chest and cough with mucoid expectoration. Temperature 97.8 F. Smears from right nostril showed a few pus cells but no bacteria.

⁸ Jour. Am. Med. Assn., 1919, 72, p. 476.

Culture from right nostril showed no influenza bacilli. Smear from left nostril showed many pus cells, gram-positive diplococci and gram-negative bacilli which stained poorly. Cultures negative for influenza bacilli. Culture from throat showed no influenza bacilli. Smears from sputum showed pus, mucus and many influenza bacilli. Culture from sputum showed a few colonies of *B. influenzae*.

April 22, 10 a. m.: Patient spent another restless night. This morning nose was still stopped up and throat sore. Complained of hoarseness and soreness in chest. Cough still present but not so severe. Headache and general malaise persisted. There was stiffness in the back when he attempted to bend over. Mucopurulent discharge from the nose. Patient still looked sick; rhinitis had improved, but throat was still red. Smear from right nostril showed many pus cells and a few intracellular influenza bacilli. Cultures

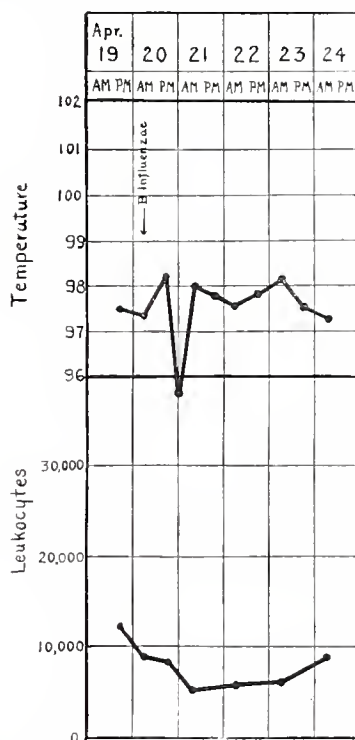


Fig. 2.—Case 5, W. J. S.; 0.5 c.c. 6-hour chocolate broth culture of *B. influenzae* in each nostril.

showed a few colonies of influenza bacilli. Smear from left nostril was similar to that of right. Cultures negative for influenza bacilli. Cultures from throat showed predominance of staphylococci, but 25% of colonies were those of *B. influenzae*. Strain biologically identical with "Wick" strain.

April 23: Patient was much better; had moderate rhinitis, no headache. Cultures from throat showed a great predominance of influenza bacillus (75%). Strain was biologically identical with "Wick" strain.

April 24: Patient's condition practically normal.

April 25: Patient was feeling well.

April 27: Patient remained well. Cultures from throat showed a large number of colonies of *B. influenzae*.

May 3: Cultures from throat still positive for *B. influenzae*. Patient felt well and throat appeared normal.

This volunteer reacted to the inoculation in a manner similar to that of the two preceding cases. The temperature was not elevated at any time but 12 hours after inoculation the temperature was 95.6. He also showed a striking leukopenia, the number of leukocytes falling from 12,000 to 6,000 (Fig. 2). The patient became an influenza bacillus carrier following the inoculation. Cultures from the throat were positive for influenza bacilli for two or more months following inoculation.

Case 6 differed from the preceding cases in that the patient was a carrier of the influenza bacillus at the time of inoculation. It seemed desirable to inoculate one carrier purposely in order to determine whether the carrier state conferred immunity against infection by this organism. The method of inoculation was similar to that employed in case 5.

CASE 6.—T. B., a man, aged 32, had never had influenza though exposed many times. He had never had pneumonia and was not susceptible to colds. He had a mild coryza about one month before, the only respiratory infection he had had during the winter, and this attack lasted only two days. A small, muscular, robust young man; nose and throat normal.

April 19: Preliminary cultures from right nostril showed considerable number of *B. influenzae* colonies. Cultures from left nostril showed that *B. influenzae* was the predominating organism on the plates. The strain was biologically different from the "Wick" strain. Cultures from the throat showed usual mouth flora. A few colonies of *B. influenzae* were present.

April 20, 10:10 a. m.: Volunteer received 0.5 cc of a 6-hour chocolate blood-broth culture of *B. influenzae*, strain "Wick," in each nostril. The throat was rubbed with a cotton swab soaked in the same culture. Culture used was the third generation on artificial medium and had been isolated from the throat of the patient in case 4. 12:30 p. m.: Patient was sneezing. 4 p. m.: Nose felt stopped up and there were drowsiness and general malaise. On physical examination the patient appeared to be in good condition; throat negative. Smear from right nostril showed a few pus cells and intracellular influenza bacilli. Cultures showed predominance of *B. influenzae*. Smears from left nostril were similar to those from right. *B. influenzae* predominated in cultures. The strain was biologically different from the "Wick" strain. Cultures from throat were unsatisfactory, not suitable for examination.

April 21, 10 a. m.: Patient reported at laboratory. Had a rather uncomfortable night on account of sneezing, discharge from nose and burning of eyes. During the night he also developed a slight cough and felt sore through his chest. In the morning his chest was still sore and he had a cough with some expectoration. General malaise was marked, and he had pain in his chest when he coughed. Smears from each nostril showed mucus, many pus cells and a moderate number of influenza bacilli, some intracellular and some extracellular. *B. influenzae* predominated in cultures. Culture from throat

showed a variety of bacteria, but the colonies of *B. influenzae* were numerous. Smear from sputum showed mucus and many pus cells with moderate numbers of *B. influenzae* and a gram-positive diplococcus.

April 22, 10 a. m.: Patient had a headache the previous evening. Nose was still stopped up. Cough persisted but was not severe. Patient continued to feel tired and drowsy but remained on duty. Soreness in chest had disappeared. Smear from right nostril showed mucus and pus and many influenza bacilli. Gram-positive diplococci also were present. Cultures showed an almost pure growth of *B. influenzae*. Smear from left nostril showed pus and a few influenza bacilli. Cultures showed *Staphylococcus albus* and *B. influenzae*. In cultures from throat *B. influenzae* colonies predominated, constituting about 50% of all the colonies.

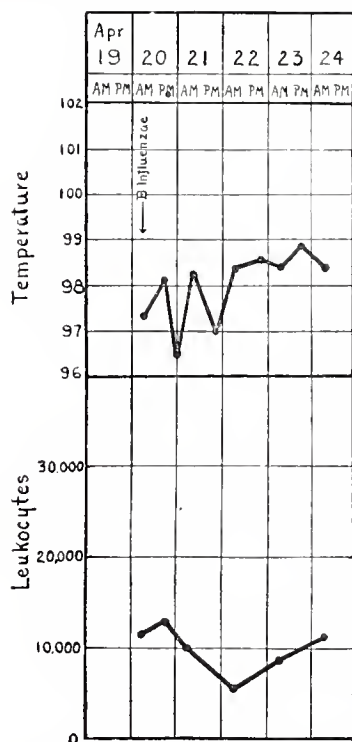


Fig. 3.—Case 6, T. B.; 0.5 c.c. 6-hour chocolate broth culture of *B. influenzae* in each nostril.

April 23: Patient was feeling well. Smears from right nostril showed pus and gram-positive diplococci. In cultures, *B. influenzae* predominated. Smears from left nostril were similar to those of right. Cultures were positive for *B. influenzae* (about 50% of colonies). Two types of influenza bacilli isolated from plates, one identical biologically with "Wick" strain, the other different. The two types were about equally represented.

April 27: Patient had entirely recovered. Culture from throat was positive for *B. influenzae*.

May 3: Culture from throat was positive for *B. influenzae*.

This patient ran a somewhat milder course than the other volunteers, and the question naturally arises whether the mildness of his

attack was dependent in any way on the fact that he was already an influenza bacillus carrier. Agglutination and absorption tests indicated clearly that the strain which he carried and the strain which was inoculated were entirely distinct biologically. It is possible, however, that there was some cross protection conferred by the strain which the volunteer carried. As in the previous cases, the temperature remained stationary and the leukocytes dropped; indeed, the temperature also dropped following the inoculation to 96.5 F. The leukocyte curve was quite similar to that observed in case 5. The patient's count before inoculation was 11,500. Following inoculation it gradually went down until it reached 6,500 on the second day of his infection (Fig. 3). This experiment would appear to indicate, however, that influenza bacillus carriers are not necessarily immune to infection by a different strain of influenza bacillus. Such a conclusion is in agreement with observations on carriers of certain other pathogenic bacteria.

INOCULATION OF VOLUNTEERS WITH FILTRATES OF BACILLUS INFLUENZAE CULTURES

In order to determine whether the symptoms produced by inoculation with *B. influenzae* were due to an infection or merely to the absorption of a toxic substance, two volunteers were inoculated with a filtrate from a young chocolate blood-broth culture of *B. influenzae*. After six hours' incubation the cultures were passed through a Berkefeld filter, and the filtrate inoculated at once into the volunteers. The same strain (strain "Wick") that was used for the preceding experiments was employed for the filtrate inoculations.

CASE 7.—F. K., a man, aged 21, had no history of influenza or pneumonia. He usually had one cold every winter; the last one in the autumn of 1919. Tonsils were removed in childhood. Healthy, well developed young man; nose and throat normal. Leukocytes 10,000.

May 2, 1920: Preliminary cultures from each nostril showed almost pure growth of *staphylococcus albus*. Cultures from throat showed *pneumococcus* type 4 and *Streptococcus hemolyticus*. No colonies of influenza bacillus on plates.

May 4, 3 p. m.: Received 0.5 c c of filtrate from a 6-hour chocolate blood-broth culture of *B. influenzae*, strain "Wick," in each nostril. The throat was rubbed with a cotton swab which had been soaked in the same filtrate. Culture used was the fourth generation on artificial medium.

May 5, 1 p. m.: Patient showed no symptoms following inoculation. Had noted a little phlegm in his throat, but smears taken from both nose and throat showed no pus cells present. Temperature 98.6 F.; leukocytes 10,700.

May 6, 1 p. m.: No symptoms except a slight headache early this morning, but he does not attribute it to the inoculation. (Subject to headaches, probably referable to eye strain.) Temperature 98.2 F.

May 7, 1 p. m.: No symptoms either local or general. Temperature 98.6 F.; leukocytes 11,000.

CASE 8.—M. K., a man, aged 25, a dental student, had no history of influenza or pneumonia; he was not susceptible to colds or sore throat. A healthy young man; nose and throat normal; leukocytes 7,000.

May 2, 1920: Preliminary cultures from nose and throat showed no colonies of *B. influenzae* on any of the plates.

May 4, 3 p. m.: Received 0.5 c c of a filtrate from a 6-hour chocolate blood-broth culture of *B. influenzae*, strain "Wick," in each nostril. In addition the throat was rubbed with a cotton swab soaked in the same filtrate. Culture used was the fourth generation on artificial medium.

May 5: Patient had developed no symptoms of any kind. Smears from nose and throat showed no pus cells present. Temperature 98.5 F., leukocytes 12,000.

May 6: No symptoms; nose and throat perfectly normal on examination; temperature 98.7 F.; leukocytes 9,300.

May 7, 1 p. m.; Patient remained well. Temperature 98.2 F.; leukocytes 11,200.

While it would be unwise to infer too much from only 2 experiments, it would appear from these experiments that filtrates of young cultures of *B. influenzae* are incapable of producing acute inflammatory symptoms in the nose and throat of healthy volunteers. It has been shown by Parker ⁸ that young cultures of *B. influenzae* contain a toxic substance, small doses of which will kill rabbits in a few hours. There was a possibility, therefore, that the symptoms produced by the intranasal injection of *B. influenzae* cultures were referable to absorption of this toxin. The negative results, however, obtained with filtrates from the cultures tend to eliminate such a possibility.

INOCULATION OF VOLUNTEERS WITH YOUNG BLOOD-BROTH CULTURES OF *STREPTOCOCCUS HEMOLYTICUS*

It seemed desirable to control the experiments with *B. influenzae* still further by inoculating some volunteers with other pathogenic bacteria. Accordingly, two volunteers who did not harbor *Streptococcus hemolyticus* in their noses or throats (2 colonies in throat of patient in case 9) were selected for inoculation with a strain of *Streptococcus hemolyticus* freshly isolated from the tonsils of a young woman with acute follicular tonsillitis and peritonsillar abscess. This organism was fairly virulent, 0.1 c c of a blood-broth culture killing a mouse in 24 hours.

CASE 9.—R. S., a man, aged 21, had no history of influenza or pneumonia. He had had two colds during the winter, the last one about two months before. He was subject to occasional attacks of sore throat but had never had ton-

sillitis. A healthy young man, well developed and well nourished; no nasal obstruction or inflammation; tonsils were large, otherwise throat was normal; leukocytes 11,000.

April 29: Preliminary cultures from right nostril showed that the predominating organisms were *Streptococcus viridans* and *Staphylococcus albus*; left nostril: *Staphylococcus albus* and a gram-negative diplococcus. Cultures from throat: The predominating colonies were pneumococcus type 4 (75% of colonies); staphylococcus colonies were fairly numerous; 2 colonies of *Streptococcus hemolyticus*.

May 1, 3 p. m.: Received 0.5 cc of a 6-hour chocolate blood-broth culture of *Streptococcus hemolyticus* in each nostril, and in addition the throat was rubbed with a cotton swab which had been soaked in the culture. This culture was the third generation on artificial medium (3 days after isolation from patient).

May 2, 10 a. m.: Patient had had no local or general symptoms of any kind. Temperature 98.2 F.; leukocytes, 9,600. Culture from right nostril showed pneumococcus type 4 and staphylococcus albus; no colonies of *Streptococcus hemolyticus*. Culture from left nostril was same as right except that the plate showed one colony of *Streptococcus hemolyticus*. Throat: At least one half of the colonies were those of *Streptococcus hemolyticus*.

May 3, 1 p. m.: Patient's throat felt a little dry in the morning when he first awoke, but after gargling this disappeared. Throat slightly red but tonsils showed no change. Temperature 97.9 F., leukocytes 11,300. Culture from each nostril showed that pneumococcus type 4 predominated; no colonies of *Streptococcus hemolyticus* were present. Culture from throat: *Streptococcus hemolyticus* predominated.

May 4: Nose and throat normal. Patient felt well. Temperature 97.4 F.; leukocytes 10,500. Cultures from nostrils were negative for *Streptococcus hemolyticus*. Culture from throat was positive for *Streptococcus hemolyticus*, but colonies were not so numerous as in culture of May 3.

May 5: Patient remained well. Temperature 98 F.; leukocytes, 10,200. Cultures from nostrils were negative for *Streptococcus hemolyticus*. Culture from throat showed that colonies of *Streptococcus hemolyticus* were still very numerous.

May 7: Culture from throat showed a few colonies of *Streptococcus hemolyticus*, but patient was entirely free from symptoms.

In this case the inoculation of a virulent *Streptococcus hemolyticus* gave practically negative results. The temperature and leukocytes remained normal. The patient had no constitutional symptoms, and locally the only change noticed was a slight dryness in the throat which lasted only a few hours. In spite of the failure, however, of the hemolytic streptococcus to produce infection, the organism remained in the patient's throat for a week, and perhaps longer, following the inoculation.

The second volunteer in this experiment (case 10) received the same strain of *Streptococcus hemolyticus* and the same amount of culture as case 9. The results, however, were more definite, as the following protocol will show.

CASE 10.—H. W., a man, aged 20, had no history of influenza or pneumonia. He had an average of about two colds a year. He had had his last cold five weeks before. He had never had tonsillitis and no nasal trouble. A robust, well nourished young man; nose and throat normal; tonsils small and appeared to be normal.

April 29, 1920: Preliminary cultures from nostrils showed staphylococcus albus. Culture from throat showed the usual mouth flora with Staphylococcus albus predominating; no colonies of Streptococcus hemolyticus on any of the plates.

May 1, 3 p. m.: Received 0.5 c.c. of a 6-hour chocolate blood-broth culture of Streptococcus hemolyticus in each nostril. Throat also rubbed with a cotton swab soaked in the same culture. The culture used was the third generation on artificial medium.

May 2: During the night the patient had a headache and was conscious of a slight soreness on the right side of the throat. The tonsils appeared somewhat reddened in the morning and there was still slight headache. Temperature 98.4 F.; leukocytes 10,000. Culture from right nostril showed 4 colonies of Streptococcus hemolyticus; Staphylococcus albus predominated. Cultures from left nostril showed pure growth of Staphylococcus albus. There were a few colonies of Streptococcus hemolyticus in the throat. Streptococcus viridans and Staphylococcus albus predominated.

May 3, 1 p. m.: Soreness in throat was more marked on left side. Felt well with the exception of a sore throat and slight headache. Temperature 100.6 F.; leukocytes 18,000. Cultures from nostrils were negative for Streptococcus hemolyticus. Culture from throat showed an increasing number of Streptococcus hemolyticus colonies.

May 4: Condition had improved, but throat was still sore. He did not sneeze or cough. Glands in neck felt a little sore. Tonsils were still inflamed and covered with a moderate amount of exudate. Cultures from nostrils were negative for Streptococcus hemolyticus. About 15% of colonies in cultures from throat were those of Streptococcus hemolyticus.

May 5: Patient felt well. Throat appeared normal. Cultures from nostrils were negative for Streptococcus hemolyticus. Three colonies of Streptococcus hemolyticus were found in throat culture.

May 7: Patient remained well.

It will be seen from this protocol that this volunteer developed an acute follicular tonsillitis following inoculation with Streptococcus hemolyticus. The infection, however, was a mild one and lasted only 3 days. There was a moderate febrile and leukocyte reaction and mucopurulent exudate over the tonsils. The patient was practically free from malaise. Headache was present, but not severe. Hemolytic streptococci persisted for 5 days, and perhaps longer, in the nasopharynx.

Summarizing these two experiments with the Streptococcus hemolyticus, it may be said that the inoculation of healthy volunteers with a virulent Streptococcus hemolyticus produced either no symptoms at all or gave rise to an acute tonsillitis with fever and leukocytosis.

INOCULATION OF VOLUNTEERS WITH A YOUNG BLOOD-BROTH
CULTURE OF PNEUMOCOCCUS

As a further control on the *B. influenzae* inoculations, two volunteers were selected for inoculation with a virulent pneumococcus. For this experiment two healthy men were chosen whose preliminary cultures showed them to be free from pneumococci. A freshly isolated, highly virulent pneumococcus group 4, was used for these inoculations. This strain had been obtained from the sputum of a child with influenzal pneumonia.

CASE 11.—H. Z., a man, aged 20, gave no history of influenza or pneumonia. He had had frequent attacks of tonsillitis in childhood, but none since 1915. He has two or three colds every year; last cold about four months ago. A healthy, well developed young man; no nasal obstruction; throat clean; tonsils small; leukocytes 10,300.

April 29: Preliminary cultures from nostrils showed *Staphylococcus albus* and *Streptococcus viridans*; usual mouth flora; no pneumococcus colonies observed on any of the plates. Mouse inoculated intraperitoneally with 0.5 cc of patient's saliva remained well.

May 1, 3 p. m.: Received 0.5 cc of a 6-hour chocolate blood-broth culture of pneumococcus type 4, in each nostril, and throat rubbed with a cotton swab soaked in the same culture. Culture used was the third generation.

May 2, 10 a.m.: Patient sneezed twice on afternoon of May 1 after receiving the inoculation; otherwise he had had no symptoms, either local or general. Temperature 98.4 F.; leukocytes 9,900. Cultures from nostrils showed *Staphylococcus albus*; from throat, *Streptococcus viridans*, *Staphylococcus albus* and a gram-negative bacillus; no pneumococcus colonies on any of the plates.

May 3: He continued to feel well. Temperature 98.6 F.; leukocytes 9,900. Smears from nostrils were negative. Cultures from nostrils showed *Staphylococcus*, *Streptococcus viridans* and pneumococcus type 4. Cultures from throat showed *Streptococcus viridans* and *Staphylococcus albus*.

May 4: No symptoms; temperature 98 F.; leukocytes 10,200. Cultures from right nostril showed *Staphylococcus albus*; from the left nostril, *Streptococcus viridans*, *Staphylococcus* and gram-negative diplococcus.

May 5: Remained well. Temperature 98.8 F.; leukocytes 9,100. Cultures from nostrils were negative for pneumococcus.

May 6 to 8: Patient remained well.

This volunteer showed no reaction, either local or general, to inoculation with a pneumococcus group 4, though the culture inoculated was highly virulent for mice and had been freshly isolated from a case of influenzal pneumonia.

A second volunteer was inoculated with the same culture of pneumococcus and he, too, failed to develop any symptoms.

CASE 12.—C. W., a man, aged 29, gave no history of influenza or pneumonia. He was not subject to colds or sore throats; last cold about three months ago.

A well developed young man; throat clean, nasal passages clear; leukocytes 8,400.

April 29, 1920: Preliminary Cultures on Blood-Agar Plates: Nostrils: *Staphylococcus albus*; throat: *Staphylococcus albus* and *Streptococcus viridans*. White mouse inoculated intraperitoneally with one-half c c of saliva remained well.

May 1, 3 p. m.: Received 0.5 c c of a 6-hour blood-broth culture of pneumococcus type 4 in each nostril; in addition the throat was rubbed with a cotton swab soaked in the same culture.

May 2, 10 a. m.: Sneezed several times May 1 just after receiving the inoculation. Developed no symptoms last night. Felt well in the morning. Temperature 98.5 F.; leukocytes 10,000. Cultures from right nostril showed *Staphylococcus albus* and pneumococcus type 4; one colony of pneumococcus type 3. Left nostril: showed *Staphylococcus albus* and pneumococcus type 4. Throat showed about 50% of colonies are those of pneumococcus type 4.

May 3, 1 p. m.: Throat felt a little dry; otherwise no symptoms. Temperature 98.6 F.; leukocytes 13,700. Smears from nostrils showed no pus cells. Cultures from nostrils showed *Staphylococcus albus*; from throat, a few colonies of *Streptococcus viridans*, *Staphylococcus albus* and pneumococcus type 4.

May 4: No symptoms in respiratory tract. He had slight indigestion; temperature 98.7 F.; leukocytes 10,400. Culture from right nostril showed predominance of pneumococcus type 4 colonies; a few colonies of pneumococcus type 3. Left nostril was negative for pneumococcus. The plate showed chiefly *Staphylococcus albus*. The throat plate was overgrown with some saprophyte.

May 5: Felt well; temperature 98.2 F.; leukocytes 9,400. Cultures from nostrils were negative for pneumococcus. Cultures from the throat showed a few colonies of pneumococcus type 4.

May 8: Remained well.

The patient case 12, as in case 11, failed to develop symptoms of a respiratory infection following the inoculation of virulent pneumococci. The negative results obtained in these two volunteers indicate that in man the inoculation of virulent pneumococci in the healthy nose and throat is not sufficient in itself to induce a pneumococcus infection of the upper respiratory tract, though no sweeping conclusion can be based on so small an experiment.

DISCUSSION

The results obtained in these experiments confirm, in almost every particular, the studies on experimental influenza recently carried out by Blake and Cecil on monkeys. In both instances, the inoculation of virulent influenza bacilli usually gave rise to an acute respiratory disease, similar in many respects to clinical influenza, and characterized by the presence of influenza bacilli in the discharges. In monkeys the hemolytic streptococcus, when inoculated in the nose and throat, caused no reaction. In man, however, one of the two volunteers developed an acute tonsillitis from which *Streptococcus hemolyticus* was recovered.

In both species virulent pneumococci failed in a few trials to excite symptoms.

In view of the failure of most of the previous experimenters to produce a respiratory infection in man with the influenza bacillus, it is interesting to speculate as to the reasons for success in our own experiments. The use of freshly isolated, virulent strains was probably an important factor. The character of the culture medium employed also appeared to have considerable influence. The first experiment with the washings from chocolate blood-agar slants was practically negative. The second experiment with the same sort of suspension was fairly successful, but a large dose (one slant in each nostril) was employed, and the symptoms produced were temporary. The two experiments with small doses of peritoneal exudate from a monkey with *B. influenzae* peritonitis were successful; and so were the last two experiments with small doses of young chocolate blood-broth cultures. It would appear, therefore, that fluid cultures were more likely to produce positive results than the washings from solid medium.

As to the character of the infection produced by these inoculations, the most that can be said is that the experimental disease closely simulated the milder forms of endemic influenza or severe coryza. The short period of incubation in the experimental disease agrees with clinical observations as to the incubation period of influenza in some cases. In respect to the local manifestations, the analogy is striking. The onset with sneezing, rhinitis and sore throat, followed in some cases by tracheitis, presents a marked similarity to influenza. The systemic reaction in the experimental disease was not so profound as in true influenza, and resembled more the prostration that accompanies a severe cold or bronchitis. It may be remarked in passing, however, that many common colds are apparently due to the influenza bacillus. Headache was noted in 5 of the volunteers. The prostration in case 3 was quite marked, and the patients in cases 4 and 5 were in bed during part of their illness. Leukopenia was present in 3 of the cases (4, 5 and 6), in the other 3, the leukocytes remained practically unchanged. Here again the findings were in harmony with clinical influenza and with the experimental infections produced in monkeys. The absence of fever in all 6 cases was rather surprising. Its absence constituted a point of divergence from typical influenzal infections and throws some doubt on the identity of the experimental disease with influenza as seen in the

epidemics. The occurrence of severe influenza without a rise of temperature is not unusual, however. The patient with the fatal case of influenzal pneumonia from which strain "Wick" was isolated, and with which 5 of the volunteers were inoculated, had been in the hospital only 48 hours when death occurred. During that time, however, the temperature did not rise above 99° F.

The influenza bacillus was recovered from the nose and throat of the 5 volunteers that developed local symptoms. In case 2, however, the influenza bacilli had disappeared 24 hours after inoculation. In the 4 remaining cases (those that received *B. influenzae* exudate or blood-broth culture) influenza bacilli persisted in the nose or throat for several days or even weeks after inoculation. Many of the plate cultures taken at the height of the infection gave almost pure growths of *B. influenzae*.

Agglutination and absorption tests were carried out by Dr. Povitsky of the Research Laboratories on the strains recovered from the patients, and they proved in every case to be identical with the strain inoculated. Cases 3 and 6 are of special interest in that they throw some light on the question of *B. influenzae* immunity. The patient in Case 3 was making rapid recovery from his infection when, following exposure to cold, he had a relapse and was more ill than he had been during the first attack. Cultures taken during the first attack showed influenza bacilli identical biologically with the strain inoculated. When, however, cultures were taken during the relapse, influenza bacilli were recovered in large numbers, but agglutination and absorption tests showed that they were of a strain entirely different from the one originally injected. Apparently, infection with one strain of *B. influenzae* gave no protection against reinfection with another strain of different type. The patient in case 6 was an influenza bacillus carrier, but was purposely inoculated with strain "Wick" to find out whether he possessed any immunity against this organism. The strain he carried and the strain inoculated were biologically unlike. Following inoculation he developed practically the same chain of symptoms, both local and constitutional, that had been observed in the previous patients, none of whom had been *B. influenzae* carriers. Furthermore, when cultures were taken on the third day after inoculation, and a number of *B. influenzae* colonies were picked from the plates, 2 strains of *B. influenzae* were recovered, the strain which had been present in the throat before inoculation and the actual strain injected. One week after inoculation, strain "Wick" had disappeared entirely from the

nose and throat and the carrier strain only survived. The conclusion may be drawn that *B. influenzae* carriers are not necessarily immune to a virulent strain of this organism.

In some of the volunteers, the cultures remained positive for *B. influenzae* a surprisingly long time after recovery from the infection. In case 3, the cultures were still positive 2 weeks after inoculation. In case 4, positive cultures were obtained more than 2 months after inoculation. The identity of these strains with the strain inoculated was, of course, verified by agglutination and absorption tests. These results were in sharp contrast with those of Bloomfield, who found that strains of *B. influenzae* isolated from healthy mouths disappeared rapidly after injection into the healthy nose and throat.

The experiments with filtrates of *B. influenzae* cultures clearly eliminate the possibility that the results obtained in these experiments might have been referable to some soluble toxic substance. Even admitting that a small portion of the toxin remained behind in the filter, this must have been an inconsiderable part of the whole, and not enough to have materially affected the result. There is abundant clinical and bacteriologic evidence that we were dealing in these cases with genuine infections.

The experiments with the hemolytic streptococcus are interesting and corroborate the findings of Richey,⁹ who noted a number of cases of streptococcus hemolyticus tonsillitis following inoculation of healthy volunteers with the washings from the noses and throats of patients with influenza. It is probable that altogether, aside from the question of virulence, the results following inoculation with *Streptococcus hemolyticus* depend largely on the source of the strain. In this particular instance, a strain isolated from a case of acute tonsillitis with peritonsillar abscess, excited an attack of acute tonsillitis in one of the volunteers inoculated with the culture.

A virulent pneumococcus of the type 4 group isolated from a case of influenzal pneumonia, was inoculated into the nose and throat of two volunteers and failed to excite any symptoms. It should not be inferred from these two experiments, however, that the pneumococcus never acts as the primary infectious agent in acute infections of the nose and throat. It is possible that pneumococcus and certain others of the bacteria that are associated with coryzas and sore throats invade the tissue only after some depression in the local or general resistance of

⁹ Jour. Infect. Dis., 1919, 25, p. 299.

the host. The recently published experiments of Grant, Mudd and Goldman¹⁰ bear out this theory. The capacity of all these bacteria to act as secondary invaders cannot be questioned; but the experiments reported in this paper indicate that virulent influenza bacilli possess a peculiar power of attacking healthy mucous membrane and of exciting a respiratory infection quite similar in some of its clinical manifestations to spontaneous influenza.

SUMMARY AND CONCLUSIONS

Virulent influenza bacilli, when injected into the nose and throat of healthy volunteers, may excite in them an acute respiratory disease, similar in many respects to influenza, but falling short of the typical clinical picture.

In such cases influenza bacilli, biologically identical with those inoculated, may be recovered from the discharges as long as symptoms persist and often for some time thereafter.

Filtrates of *B. influenzae* cultures, when similarly injected into two healthy volunteers, produced neither local nor constitutional reaction.

The inoculation of healthy volunteers with virulent hemolytic streptococci may in some cases induce an acute follicular tonsillitis, with fever and leukocytosis. A virulent pneumococcus type 4 on the other hand, was injected into the nose and throat of two healthy volunteers with impunity.

¹⁰ Jour. Exper. Med., 1920, 32, p. 87.

BOTULISM FROM CHEESE

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On Oct. 19, 1914, a sample of home-made cottage cheese was sent for examination to the Laboratory of the New York State Department of Health, by Dr. Charles G. Duryea, sanitary supervisor. Three persons who had eaten the cheese had died. The clinical data furnished by Dr. Willard Gillette and Dr. Charles G. Duryea suggested different diagnoses, including poliomyelitis, and a definite diagnosis was not made. Two cases were identical; paralysis of the muscles of deglutition, suffusion of the face, ptosis, total dilatation and failure of pupils to react to light, paralysis of the muscles of the throat with difficult speech were present. There was no loss of consciousness or paralysis of any other part of the body. The third patient was unable to swallow, and in the throat a considerable quantity of mucus accumulated. No eye symptoms were evident, doubtless owing to the fact that it was a milder infection, as the death occurred several days after that of the other two patients.

It is noteworthy that Dr. Duryea, in his search of the literature, recognized that the clinical picture in these patients corresponded to that described by Ostertag, van Ermengem and other investigators of botulism.

Necropsy examination was made and Dr. E. Kellert of the Bender Laboratory, Albany, reported:

Specimen consists of the brain of an adult and a small fragment of spinal cord from the upper cervical region. The specimens are received in a tin pail containing salt solution. The cortical vessels are deeply injected. At the base of the brain the pia appears to be slightly thickened and opaque. No exudate or gross lesion is visible.

Sections from the pons and cord show no cellular infiltration or lesion about the vessels. The white matter appears normal. Many of the ganglion cells are pale-staining, slightly shrunken and have irregular margins. The nuclei are very pale or absent. The Nissl bodies in certain of the cells are pale or absent. In the immediate vicinity of such cells there is an accumulation of numerous granules varying greatly in size and staining deeply with methylene blue and hematoxylin. A few lymphocytes are present in the region of the anterior horns.

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Cultures from the ventricles showed a mixed infection in which *B. coli* predominated. Stained sections from the cord and pons were referred to Dr. Simon Flexner of the Rockefeller Institute, who found no evidence of poliomyelitis. About 30 c c of bloody fluid from the skull were also inoculated by Dr. Flexner into monkeys with negative results.

Bacterial Examination of the Cheese.—When the sample of cheese reached the laboratory, emulsions were made and inoculated into guinea-pigs and at the same time cultures to isolate the bacteria were made. It was found that the subcutaneous inoculation of 3 c c of an emulsion of the cheese after incubation at 37 C. for 48 hours, killed guinea-pigs within 36 hours. Attempts to isolate the bacteria, which might have been the causative agents, were then made. Under aerobic conditions, a staphylococcus, *B. coli* and *B. subtilis* were obtained. In anaerobic cultures another organism was found. After heating the emulsion of the cheese at 85 C. for 10 minutes, only two organisms were found in anaerobic agar cultures of the material. One of these was *B. subtilis* found in the aerobic cultures and the other a bacillus, also with spores, but which developed colonies only in anaerobic cultures, which were distinctive.

This organism, somewhat smaller than *B. subtilis*, was a motile bacillus with rounded ends, and was gram-positive, except in older cultures, when it often decolorized. Oval spores developing early were situated about the end of the middle third. In the adult stage these spores appeared to be terminal. The thermal death point was found to be 80 after one-half hour of exposure and 85 C. after 15 minutes of exposure. The organism had no demonstrable capsule in vitro or in vivo.

Gelatin was liquefied slowly and milk coagulated in three days; dextrose, galactose, levulose, lactose, saccharose, maltose, mannit, dextrin, inulin and glycerine were fermented with the production of gas and the odor of butyric acid, which was marked.

According to van Ermengem,¹ the strain from Ellezelles did not coagulate milk, but von Hibler found that the strain of *B. botulinus* from Ellezelles, the Damstadt strain and a third isolated from meat often precipitated and peptonized casein, with energetic gas production. The Ellezelles strain that von Hibler worked with had lost its toxicity. Van Ermengem and others observed that the addition of peptone to the medium was necessary for the growth of *B. botulinus*.

¹ Kolle and Wassermann: Handb. d. path. Mikroorg., 1912, 4, p. 920.

On the contrary, the writer found that potent toxins were obtained in the following medium, made without the addition of peptone:

Chopped veal	1,000 gm.
H ₂ O	1,000 c c
NaCl	0.5%
Dextrose	2.0%
Reaction adjusted 0.3-0.5 alkaline to phenolphthalein.	

Mice, guinea-pigs and rabbits were killed by the inoculation of cultures of this organism and also by Berkefeld filtrates of the cultures, which were found to be equally toxic.

No special study was made of the conditions under which the more potent toxins were produced. However, contrary to recorded observations of other investigators, that the bacillus forms toxin and spores only when incubated below 30 C., the bacillus not only grew luxuriantly in medium containing dextrose, at 37 C. in from 18 to 24 hours and developed spores in 48 hours, but it also produced potent toxins. Guinea-pigs were killed in 4 days with 0.0005 c c of the filtrate of a 72 hour culture.

After inoculation, a period of incubation was followed by loss of accommodation, partial paralysis of the hind legs, dragging of the abdomen; hypersecretion of mucus from the nose and mouth and accelerated respiration were noted, but no fever. No pigs developing these symptoms recovered. At necropsy, a slight congestion was noted at the point of inoculation, and in animals which received cultures the bacillus was found in the edema which was present. Varying degrees of congestion of the lymphatic glands were found and also of the suprarenals, such as is seen after the inoculation with diphtheria toxin. The reaction induced by pure cultures, as compared with those obtained from culture filtrates, differed only at the site of inoculation. In all the animals used, only once was the bacillus found in an organ, namely, in the liver.

In order to obtain the classic picture described by van Ermengem, a kitten weighing 720 gm. was inoculated subcutaneously with 5 c.c. of toxin. Within 36 hours, it developed marked symptoms of botulism: hypersecretion from nose and mouth, paresis of the hind legs, mydriasis and intermittent hiccoughs. In 72 hours, the symptoms had developed so far that the animal was unable to move from its side. Partial paralysis developed in the front legs, the pupils were dilated and showed but slight response to light; all food was refused. The weight of the animal dropped from 720 to 565 gm., and it was chloro-

formed on the fifth day. At necropsy, congestion and degenerative changes in the parenchyma of the organs, notably of the liver, were found and retention of the urine and feces.

For the final identification of the organism, cultures of *B. botulinus* from the Museum of Natural History and from the Medical School of Harvard University, were carried through the different mediums with this organism for the purpose of comparison. The cultural characteristics corresponded in all particulars, but guinea-pigs inoculated with 3 c c of a 48-hour broth culture of both these strains failed to develop any evidence of intoxication.

Although the early investigators thought that botulinus toxin was produced only in the presence of meat protein, it has been found that the poison is produced in the presence of vegetable protein (Dickson²). In my study, I found that *B. botulinus* grew and produced toxin in ordinary cottage cheese. An emulsion of fresh cottage cheese was sterilized, inoculated with one loopful of a 48-hour broth culture of the organism and incubated at 37 C. for 72 hours. A guinea-pig injected with 1 c c of the emulsion died in less than 15 hours. Post-mortem examination showed that the veins of the subcutaneous tissues were intensely congested, radiating from the point of inoculation to the axillae and groin. The liver was mottled and the suprarenals hyperemic.

The same technic was followed with cheese not sterilized. The guinea-pig inoculated with 1 c c died in 2½ days, showing the same picture. For purposes of control, an emulsion of market cheese which had not been sterilized was incubated for 72 hours at 37 C. A guinea-pig inoculated with 1 c c showed no evidence of intoxication.

Further studies of the toxin and its action on the tissues were carried out with a view to studying the development of antitoxin in the animal body. Van Ermengem observed a certain degree of resistance in cats and rabbits so that they withstood more than the lethal dose of toxin for other animals, such as the guinea-pig. But all these animals died with cachectic symptoms, and no marked immunity was induced. Tschitschkine,³ by giving small quantities of toxin by mouth, succeeded after great difficulty and the loss of numerous rabbits, in inducing in the rabbit a resistance against a fatal subcutaneous dose. This serum possessed but slight antitoxic value.

² Jour. Am. Med. Assn., 1915, 65, p. 492.

³ Ann. de l'Inst. Pasteur, 1905, 19, p. 335.

Forsman⁴ records the best results in the immunization of small animals by injecting a toxin which was heated to 60 C. for 35 minutes. The immunized animals withstood one hundred times the lethal dose. Their blood serum was highly antitoxic.

Larger animals (goats and horses) were first immunized by Kempner,⁵ then by Forssman and Lundström⁶ and more recently by Leuchs.⁷

In my attempts to immunize rabbits with the toxin, 3 animals were lost before any successful results were obtained. The fourth rabbit was inoculated every seventh day and with gradually increasing doses beginning with 0.001 c.c. The first 3 inoculations were intravenous, the next 8 were intraperitoneal. At this stage the animal was withstanding 0.3 c.c. of a toxin the M L D of which for a 300 gm. guinea-pig was 0.009, and 0.04 of which had killed a rabbit on the fourth day. From this point, a modification of Forssman's method was used. Instead of heating the toxin at 60 C. for 35 minutes, it was put in the water bath and the water brought to 60 C. After cooling, 1 c.c. of the toxin was inoculated subcutaneously into the rabbit. The animal withstood an intravenous inoculation of 5 c.c. of this heated toxin. At its last inoculation it was given subcutaneously 35 c.c. of this heated toxin, the minimal lethal dose of which when fresh was 0.01 c.c. for a 325 gm. guinea-pig. The blood serum of the animal was found to possess antitoxic value, 1 c.c. protecting a guinea-pig against 100 times the minimal lethal dose of toxin.

Shortly after this paper was read before the American Public Health Association at Rochester in September, 1915, this strain of *B. botulinus* which I had isolated was sent, on request, to the Department of Agriculture at Washington, and used in the experimental work on forage poisoning by Buckley and Shippen.⁸ Shippen⁹ used this strain exclusively in his work on toxin production, because other cultures obtained from Dickson (originally from Zinsser and the New York Museum of Natural History) produced no toxin.

I have done no work on the typing of this organism, but Burke¹⁰ and others have found my strain to be of type B, conforming to the strains subsequently isolated in the eastern part of the United States and those obtained from Germany.

⁴ *Centralbl. f. Bakteriöl.*, I, O., 1905., 38, p. 463.

⁵ *Ztschr. f. Hyg. u. Infektionskr.*, 1897, 26, p. 481.

⁶ *Ann. d. l'Inst. Pasteur*, 1902, 16, p. 294.

⁷ *Ztschr. f. Hyg. u. Infektionskr.*, 1910, 65, p. 60.

⁸ *Jour. Am. Vet. Med. Assn.*, 1916, 50, p. 809.

⁹ *Arch. Int. Med.*, 1919, 23, p. 346.

¹⁰ *Jour. Bacteriol.*, 1919, 4, pp. 541 and 555.

SUMMARY AND CONCLUSIONS

Three fatal cases presented the typical picture of botulism, and *B. botulinus* was isolated from the cheese eaten by them.

It is evident from these results that meat protein is not necessary to the growth and consequent toxin production by *B. botulinus*, and that cheese may serve as a medium.

There is little significant variation in the cultural characteristics from the type, but contrary to the observations of van Ermengem and others, *B. botulinus* grows luxuriantly and produces spores and toxin at 37 C. The addition of peptone to the medium used is not necessary as in this work potent toxins were produced without the addition of peptone.

A protective serum was produced in rabbits against the homologous toxin. It has been impossible, however, to test its antitoxic value with toxins from heterologous strains of the organism as the strains procured failed to produce toxin.

This appears to be the first time that *B. botulinus* has been isolated from cheese, that a soluble bacterial toxin has been detected in cheese, and that *B. botulinus* has been isolated in America.

THE ACIDFAST ACTINOMYCETES

WITH A REPORT OF A CASE FROM WHICH A NEW SPECIES
WAS ISOLATED

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Acidfast actinomycetes are not common and therefore not as well known as other pathogenic varieties. They are, however, of great interest from the standpoint of bacteriology, since they furnish the closest contact between the true bacteria and higher fungi, being closely related to the tubercle bacillus in morphologic and cultural characters, pathogenicity and serum reactions; and, second, from the standpoint of clinical medicine, since they produce infections that bear a great resemblance to tuberculosis, with which they may be confused, the confusion extending even to the microscopic examination of the sputum.

These infections differ from tuberculosis, however, in having a distinctly more unfavorable prognosis, the organisms being more virulent both for man and laboratory animals.

Acidfast actinomycetes form a well defined group containing several species which resemble each other closely, but may be distinguished from each other by differences in cultural characters and pathogenicity for animals. They may be distinguished from the organisms of lumpy-jaw and ordinary actinomycosis in man because they do not form clubs at the extremity of the mycelium in infected tissues, either in man or ordinarily in laboratory animals (although under certain circumstances, when the virulence is low, or in certain tissues, as in the kidneys, ray-like masses with clubs at the periphery have occurred in animals inoculated by Nakayama,¹ MacCallum,² and Lubarsch³). The acidfast actinomycetes may also be distinguished by their acid-fastness, by the readiness with which they can be cultivated, and by their cultural characters; and by their pathogenicity for laboratory animals, especially guinea-pigs.

In morphology, the various species resemble each other closely: The acidfast property was not recognized by the various bacteriologists who

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¹ Arch. f. Hyg., 1908, 58, p. 207.

² Centralbl. f. Bakteriol., I, 1902, 31, p. 529.

³ Ztschr. f. Hyg. u. Infektionskrankh., 1899, 31, p. 187.

first described these organisms, and was apparently first observed by Berestneff,⁴ and has been extensively studied by Feistmantel.⁵ Fuchs⁶ found a number of species to be at least in part acidfast. In sputum the fragments of mycelium may readily be demonstrated by the ordinary stains for tubercle bacilli; they are, however, less acidfast than tubercle bacilli, and may be decolorized if the acid is allowed to act too long. In tissues they cannot be readily demonstrated by the Ziehl-Neelson stain. Very young cultures are not acidfast, a condition also observed in cultures of tubercle bacilli; while in old cultures only certain fragments retain the stain. Feistmantel believes the acidfastness bears no relationship to fats, as is the case with tubercle bacilli.

In their cultural characters they also resemble each other closely, producing on solid medium a dry, wrinkled growth varying from a chalky white through the shades of yellow to a deep orange color, the latter appearing exactly like a culture of tubercle bacilli. The pigment depends in part on the species, in part on the nature of the culture medium and in part on the age of the culture. In liquid medium they form a wrinkled pellicle, such as is produced by tubercle bacilli. With certain exceptions to be noted later, they do not produce active ferments against either proteins or carbohydrates. They grow most luxuriantly on rich mediums containing meat extract and carbohydrates, but show some growth on practically all mediums, some varieties finding nutrition even in tap water.

It is probable that the acidfast actinomycetes occur as saprophytes in soil or on vegetable matter, as is the case with other members of this genus, and that infection occurs through spores that gain entrance to the body by inhalation or in wounds. We have found no record, however, of members of this group being isolated from such sources. Fortineau and Soubrane⁷ have described a variety of actinomycetes, isolated by Rappin from the filter beds in Nantes, which was acidfast. It produced a rose to vermilion pigment and produced localized nodules at the point of injection in guinea-pigs. One of us cultivated an organism apparently identical with this from an agar plate exposed to air; this organism was kept for some years without its acidfastness being suspected.

Several species have been isolated from diseases in domestic animals. These are *A. farcinica* (Nocard)⁸ producing a disease of cattle, *A. caprae* (Silber-

⁴ Dissertation, Moscow, 1897, quoted by Schabad.

⁵ Centralbl. f. Bakteriöl, I, 1902, 31, p. 433.

⁶ Centralbl. f. Bakteriöl., I, 1903, 33, p. 649.

⁷ Proc. Congrès International de la Tuberculose, 1905, 1, p. 177.

⁸ Ann. de l'Inst. Pasteur, 1888, 2, p. 293.

schmidt)⁹ isolated from a lung abscess in a goat, *A. canis* (Levy)¹⁰ producing infections in dogs, and an unnamed variety isolated from a guinea-pig by Zimmermann.

A. farcinica (Nocard)⁸ produces a disease of cattle known as "farcin du boeuf," which occurs sporadically in France and is apparently endemic on the island of Guadeloupe in the French West Indies. It begins as a chronic indurative lymphangitis and lymphadenitis of the subcutaneous tissues, usually on one of the extremities, the lesions developing into cold abscesses which break through, forming sinuses. It is of long duration, but leads to general wasting of the animal, eventually to pulmonary involvement and finally to generalized metastases and death. Cultures were easily obtained on all mediums. On solid mediums a pale yellowish-white wrinkled growth occurred, the surface being dry and powdery. Growth in broth occurred, chiefly at the bottom of the tube in the form of whitish granules, though some colonies floated on the surface like "globules of fat on cold soup." There was no growth on gelatine at room temperature, and no change in milk. Inoculated intraperitoneally into guinea-pigs, death occurred in from 9 to 20 days; the peritoneal surfaces were found studded with miliary nodules, the omentum particularly being a tangled mass of nodules. Injected intravenously, there was produced a condition resembling generalized miliary tuberculosis. Injected subcutaneously, the organisms formed a local abscess which drained for a long time; but extension of the infection never occurred. The cow and sheep proved more resistant, but succumbed eventually. Rabbits, cats, dogs, the horse and ass were not susceptible.

The acid-fastness of this species was apparently first demonstrated by Berestneff,⁴ who compared it with other varieties from human sources. It is noteworthy that subcultures of the original strain have maintained their pathogenicity for a long time, guinea-pigs being susceptible after 14 years (Feistmantel),⁵ while miliary nodules were produced in monkeys after nearly 20 years (Musgrave and Clegg),¹¹ computing from the dates of publication.

A. caprae (Silberschmidt)⁹ was isolated from a lesion in the lung of a goat, diagnosed as tuberculous by the veterinarian from whom Silberschmidt obtained the culture. It appeared in the form of long, tangled, branching threads, which stained readily by the more intense aniline dyes in smears, but were not readily stained in sections. In cultures the threads soon disintegrated into short bacillary and coccoid forms. On agar small dry, warty, whitish brown colonies were produced which early became covered by a whitish powder; on potato the growth was similar. On broth small white colonies in the form of concave disks were formed, which ultimately coalesced to form a thick pellicle. On milk a pellicle with a rosy tint occurred; the milk remained unchanged. Gelatine was not liquefied. Rabbits were not very susceptible, only one small animal dying after intravenous inoculation; if killed some days after inoculation, miliary nodules were found throughout the body; microscopically these resembled tubercles, giant cells being found in some. Guinea-pigs died in 4 to 6 days when inoculated intraperitoneally; miliary nodules were found on the peritoneal surfaces. Mice were not susceptible.

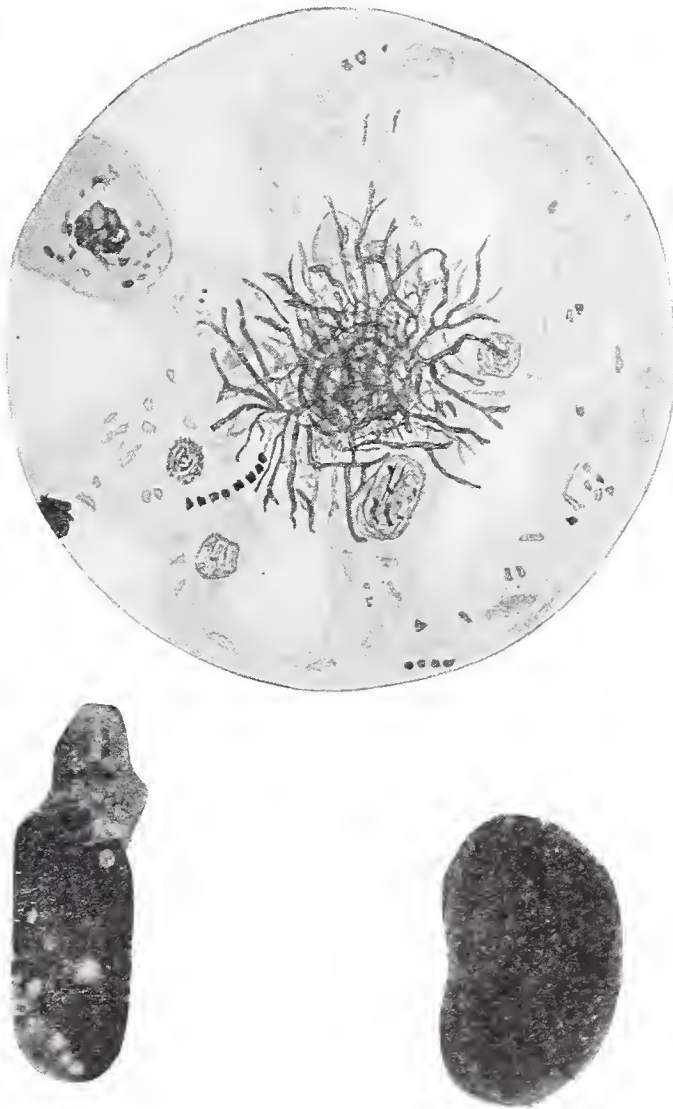
Silberschmidt⁹ made a comparative study of his organism with various other actinomycetes, and believed it most closely resembled *A. farcinica*, differing.

⁹ Ann. de l'Inst. Past., 1899, 13, p. 841.

¹⁰ Centralbl. f. Bakteriol., I, 1899, 26, p. 1.

¹¹ Phil. Jour. Sc., 1907, 2 B, p. 477.

however, in the earlier fragmentation of the mycelium, the growth on the surface of broth, and the growth on milk. In a comparative study with subcultures some years later Musgrave and Clegg¹¹ found these characters constant. The acidfastness of *A. caprae* was apparently first discovered by Fuchs.⁸



Figs. 1, 3, 4

Fig. 1.—Mass of mycelium in sputum stained by Ziehl-Neelson method. Camera lucida.

Fig. 3.—Pseudotubercle on surface of spleen. Guinea-pig inoculated intraperitoneally.

Fig. 4.—Miliary abscesses on surface of kidney. Rabbit inoculated intravenously.

Musgrave and Clegg¹¹ obtained miliary nodules in the peritoneum of monkeys with subcultures.

Rabe¹² found, in two cases of phlegmon and one of peritonitis, in dogs, a filamentous fungus which he named *Cladothrix canis*. Cultures were not made.

¹² Berlin Tirrarztl. Wchnschr., 1888 (quoted by Levy.)¹⁰

Levy¹⁰ believed this organism to be identical with *Discomyces canis* described previously by Rivolta, and with an organism isolated in his laboratory by Lange and Manasse, which he named *A. canis*. The latter was obtained from a suppurative phlegmon of the neck in dogs, and proved pathogenic on reinoculation into dogs. Musgrave and Clegg studied a subculture of this organism obtained from Levy. They found it acid fast and pathogenic for monkeys and guinea-pigs, producing miliary tubercle-like nodules on the peritoneum. In its cultural characters it proved identical with *A. caprae*.



Fig. 2.—Culture on Sabouraud agar 5 days old.

Trolldenier¹³ observed an infection in a dog which resembled many of the human cases, the primary infection being in the peribronchial lymph nodes which were caseopurulent, the dog dying from secondary brain abscesses. Smears showed acidfast branching filaments. Cultures were successful, the colonies on agar being dry and white. He believed his strain to be closely related to, but not identical with, *A. asteroides* (Eppinger¹⁴).

Zimmermann¹⁵ obtained a culture from the caseous nodules on the peritoneal surfaces of a guinea-pig previously inoculated with a myxomycete (*Plasmodiophora carassibe*). It grew in the form of white, sugary colonies on

¹³ Ztschr. f. Tiermed., 1903, 7, p. 81.

¹⁴ Beitr. zur path. Anat. (Ziegler's) 1890, 9, p. 287.

¹⁵ Pester med-chir. Presse, 1903, 39, p. 704.

agar, which later turned yellow and finally rusty. The growth on potato resembled that of the tubercle bacillus. Smears showed acidfast, branching filaments. It proved pathogenic for dogs, rabbits, guinea-pigs, mice, chickens and frogs. Inoculated intravenously, it killed rabbits in 3 to 5 days; intraperitoneal injections killed guinea-pigs in 4 to 6 days. The peritoneal surface was studded with pseudotubercles which contained no giant cells. He believed his strain was similar to Eppinger's¹⁴ *A. asteroides*, but that it differed in certain particulars, especially in its greater virulence.

Several varieties of acidfast Actinomycetes have been isolated from a number of cases in man. The first of these was isolated and reported by Eppinger.¹⁴ He named his organism *A. asteroides*. Eppinger's organism has been considered the type species of the group, and has been extensively studied. A number of cases have subsequently been reported, from which organisms have been isolated which were identical with *A. asteroides*, while from others have been obtained similar acidfast actinomycetes that differed in certain respects from Eppinger's original strain, and which should be considered as separate species; these, however, have not received specific names.

Eppinger's patient was a man 52 years old, a glassworker, who had rheumatic symptoms, but who soon became delirious and died after two weeks. The clinical diagnosis was meningitis. Necropsy revealed multiple brain abscesses with meningitis, caseous and calcareous peribronchial lymph nodes, and miliary "pseudotuberculosis" of the lungs and pleura; there were diverticula of the esophagus.

From the brain abscesses and meningeal pus the organism was isolated in pure culture. It was found microscopically in decalcified sections of the bronchial lymph nodes. In smears it appeared as long tortuous gram-positive filaments, showing branching which Eppinger considered "false," and coccoid and bacillary forms which showed motility. It has since been shown that Eppinger was in error regarding the false branching and motility.

Cultures were readily obtained on ordinary mediums. In agar the colonies appeared after a day or so as small star-like masses on account of the radiating filaments. Because of the apparent false branching and the star-like colonies, Eppinger named the organism *Cladothrix asteroides*. It has since been generally known as *Streptothrix Eppingeri*, but should be correctly termed *Actinomyces asteroides*. On solid mediums the growth appears as a wrinkled, dry mass, at first whitish, but rapidly turning yellow and later orange, old cultures on potato becoming brick red. On older cultures there appears a white, powdery layer formed by the spores. The growth on solid mediums, especially on dextrose or glycerine agar, bears a close resemblance to that of tubercle bacilli. On liquid mediums a thick, wrinkled, yellowish-orange pellicle is formed. Gelatine is not liquefied, and there is no change in litmus milk.

Rabbits inoculated intravenously died in 5 to 7 days with multiple abscesses throughout the body. Guinea-pigs inoculated intraperitoneally died in from 5 to 24 days, showing multiple miliary "pseudotubercles" on the peritoneal surfaces.

Other cases in which Eppinger's organism was the cause of disease, primary in the lungs, have been reported by Aoyama and Miyamoto,¹⁶ Horst,¹⁷ Schabad,¹⁸ MacDonald,¹⁹ Stokes,²⁰ and Loehlein.²¹

¹⁶ Mitt. a. d. Med. Fac. d. Kan. Univ., Tokyo, 1900, p. 231.

¹⁷ Ztschr. f. Heilkunde, 1903, 14, p. 157.

¹⁸ Ztschr. f. Hygiene u. Infektionskrankh., 1904, 47, p. 41.

¹⁹ Scottish Med. & Surg. Jour., 1904, 14, p. 305.

²⁰ Am. Jour. Med. Sc., 1904, 128, p. 861.

²¹ Münch. med. Wchnschr., 1907, 54, p. 1523.

Aoyama and Miyamoto's¹⁶ patient was a Japanese pawnbroker, 35 years old. Illness began with symptoms of an apoplectic stroke. Two months later a cough developed, and after a week's illness death occurred. Necropsy revealed a caseous pneumonia with an abscess cavity and an aneurism of the anterior communicating artery. Sections of the abscess wall showed a small round cell infiltration, but no giant cells. Acidfast actinomycetes were found in smears from the sputum and from pus in the abscess cavity obtained postmortem. None was found in sections of the aneurism which was probably an accidental lesion.

Cultures were readily obtained. On solid mediums the growth was at first grayish, but soon became chalky-white, later turning yellow. On liquid medium a pellicle formed. Growth on gelatine was very poor. No change occurred in milk. Guinea-pigs died in from 4 to 20 days, depending on the dosage and mode of injection. They developed a pseudotuberculous peritonitis, or a hemorrhagic-fibrinous serositis.

The authors made extensive studies of the morphology and resistance of their organism. They found it gram-positive and acidfast. Methylene blue stained the protoplasm but left the membrane clear. Irregularities in the staining of the protoplasm were due in part to plasmolysis, and in part to the presence of chromatin granules. Older cultures underwent fragmentation, and the bacillary fragments were more resistant to heat and drying than the young mycelium.

The organism did not lose its virulence for guinea-pigs after long cultivation.

Aoyama and Miyamoto¹⁶ also observed another case of caseous pneumonia in which an acidfast filamentous organism was present. A complete description, which they promised, was apparently never published.

Horst¹⁷ observed multiple brain abscesses following suppuration of a peribronchial lymph node in a carpenter aged 33. There was also a tuberculous ulcer at the ileocecal valve and caseous mesenteric lymph nodes. The cultures obtained resembled closely those of Eppinger and of Aoyama and Miyamoto. The organism was pathogenic for guinea-pigs, less so for rabbits, and not at all for mice.

Schabad's¹⁸ patient was a man 62 years old, a worker on a ship used for the transportation of grain. He suffered from a cough of 3 years' duration, and had had bloody sputum 6 months previous to admission. He was admitted to the hospital for a tumor mass on the right side of the chest, which proved to be a cold abscess. Acidfast branching filaments were found in sputum and in aspirated pus. Death occurred 5 days after admission.

Necropsy revealed that the infection had begun in a peribronchial lymph node, and was followed by a pulmonary abscess. The latter had broken into the pleura, producing an empyema, and had then eroded the third rib and produced a massive subpectoral abscess.

In its cultural characters the organism was identical with Eppinger's original strain. The results of animal inoculations were also similar to those reported by Eppinger. Pigeons were not susceptible.

MacDonald's¹⁹ patient was a charwoman 65 years old. She had been sick for half a year, was admitted in coma and died the next day. A complete history was not obtained. Necropsy revealed a caseous pneumonia with cavity formation in the lower lobe of the left lung, and secondary abscesses in the lungs, kidneys and brain. Miliary nodules were present on the pleurae. Branching filaments were found in sections of all the lesions; they could not be

demonstrated by Ziehl's stain, but stained readily with Gram's method. Sections of recent lesions in the lungs showed bronchopneumonia, the exudate being composed of fibrin and pus cells; in the older areas there was some fibrosis, with mononuclear leukocytes, but no giant cells.

In cultural characters and pathogenicity for laboratory animals the organism was identical with Eppinger's¹⁴ strain. Young cultures were not acid-fast, but older cultures undergoing fragmentation were.

Stokes²⁰ observed a pulmonary infection with *A. asteroides* in a male infant 28 days old. There was an abscess cavity the size of a pea in the lower lobe of the left lung. Smears showed acidfast branching filaments, and cultures were obtained, which proved to be identical with Eppinger's organism in all particulars.

Loehlein²¹ reported a case of multiple brain abscesses, with exudate in the ventricles, in a man 58 years old. There was also bronchiectasis and diseased peribronchial glands, and although the fungus could not be demonstrated in the latter, Loehlein believed that they were the site of the primary infection. The organism isolated was like the *A. asteroides*. It was pathogenic for rabbits and guinea-pigs, but not for dogs.

Three other cases of infection with *A. asteroides* have been reported, in which the primary infection was elsewhere than the lungs. Two of these were primary infections of the peritoneum following laparotomy, the third being a case of Madura foot.

MacCallum's² patient was a child of 3 years old suffering from stricture of the esophagus following the accidental swallowing of lye. Three weeks after a gastrostomy the child died of generalized peritonitis. *A. asteroides* was isolated in pure culture. This strain proved pathogenic for rabbits, dogs and guinea-pigs.

Rabbits were less susceptible than guinea-pigs, living 10 days or more following intravenous injection. In some of the abscesses formed in these rabbits the fungus was arranged in irregular masses about a core of calcium carbonate; the peripheral filaments of these masses showed clubbed extremities. Giant cells occurred in some of the lesions in rabbits.

Foulerton²² obtained his culture from the peritoneal exudate of a man 55 years old on whom an appendicostomy had been performed for the purpose of feeding; the patient was suffering from a carcinomatous stricture of the esophagus. There was a retrocecal abscess and a secondary abscess in the lung. Death occurred 24 days following operation.

These two cases show a remarkable parallelism. In both the peritoneum was healthy at the time of operation; the organisms then must have been introduced during the operation, probably from spores floating in the air. It is a noteworthy coincidence that both cases were suffering from esophageal strictures. Eppinger's original case also showed esophageal stricture with diverticula.

A case of Madura foot has been reported by Musgrave and Clegg.¹¹ The patient was a Philippino woman, 30 years old. The infection was of 3 years' duration. Amputation was followed by recovery. In the pus the organism appeared as yellowish granules, which when crushed and examined unstained failed to show the clubbed extremities characteristic of the Madura foot parasite. Its cultural characters were similar to those of *A. asteroides*. It proved pathogenic for dogs, monkeys and guinea-pigs; rabbits and pigeons were not susceptible. Injected into the foot of a monkey, a typical mycetoma developed.

The authors named this organism *Streptothrix freeri*, but in a later paper recognized its identity with *A. asteroides*.

²² The Lancet, 1910, I, pp. 551, 626 and 769.

In all of these cases the organisms were practically identical in all features. The strain isolated by Aoyama and Miyamoto¹⁶ differed in that the growth was chalky white and only turned yellow after some time, never becoming orange. With all of these strains the guinea-pig showed the greatest susceptibility, the rabbit being next; dogs were infected by some strains, not by others. None of the strains liquefied gelatine or digested the casein in milk.

Other cases have been reported, however, in which the organisms isolated differed in some respects from the variety described by Eppinger.

Birt and Leishman²³ cultivated an acidfast filamentous organism from the pleural pus of a South African soldier. The patient, 26 years old, became ill during the siege of Ladysmith and died 6 months later, 11 days following resection of a rib for empyema. Acidfast rods and segmented branching filaments were found in the sputum, and the organism was isolated in pure culture from pus aspirated from the chest. Necropsy revealed a chronic broncho-pneumonia, without cavity formation, in the right lung. Both lungs were studded with small cirrhotic nodules.

The colonies in agar appeared like small balls of cotton. On agar slants the growth was at first snow-white, later becoming a delicate coral pink color. Gelatine was not liquefied, but milk was slowly peptonized. On broth a pellicle was formed. Guinea-pigs inoculated intraperitoneally died in 5 to 6 weeks with caseous masses in the omentum.

This strain differs from *A. asteroides* in the absence of orange pigment, the peptonization of milk, and the lower virulence for guinea-pigs. Birt and Leishmann also found that their strain would grow on ordinary tap water, whereas Eppinger's strain did not.

Bernstein's²⁴ organism was characterized by a pure white growth, which on solid mediums formed hard masses and on liquid mediums a thick pellicle adherent to the walls of the culture tube. It did not liquefy gelatine; the action on milk is not described. It was very pathogenic for rabbits and guinea-pigs, one of the former developing endocarditis following intravenous injections, the latter developing lesions in the testicles in addition to miliary nodules on the peritoneal surfaces. Mice were not susceptible.

This organism was isolated from a suppurating localized pneumonia in the lower lobe of the right lung of a carpet worker aged 67. There was also generalized miliary tuberculous peritonitis; sections of the nodules on the peritoneum showed typical tubercles with acidfast bacilli. Bernstein is unable to state whether these were true tubercle bacilli or fragments of mycelium.

A third variety comprises the strains isolated by Ferré and Faguet,²⁵ Sabrazés and Riviére,²⁶ Berestneff,⁴ and Scheele and Petruschky.²⁷ These differed from those previously described in that they liquefied gelatine and were nonpathogenic for laboratory animals; some of them grew anaerobically.

²³ Jour. Hyg., 1902, 2, p. 120.

²⁴ Proc. Roy. Soc. of Med., 1908-1909, 2, P. 3, p. 271.

²⁵ Semaine méd., 1895, p. 359.

²⁶ Semaine méd., 1895, p. 383.

²⁷ Verhandl. d. 15. Cong. f. inn. Med., 1897, p. 550.

Ferré and Faguet isolated a branched filamentous organism from a brain abscess. The cultures on solid mediums were gray-white without any whitish bloom on the surface. Neither rabbits nor guinea-pigs were susceptible.

Sabrazés and Riviére²⁶ saw two cases, the first a brain abscess with a suppurating infarct in the kidney, the second a case of bronchopneumonia with multiple miliary subcutaneous abscesses. The organisms from these two cases were identical. The growth on solid medium was yellowish. They grew best under aerobic conditions, but showed some growth anaerobically. Gelatine was liquefied. They were not pathogenic for laboratory animals, but showed some pathogenicity when lactic acid was added to the material injected.

Berestneff's⁴ patient died of multiple cerebral abscesses, one of which had perforated into the lateral ventricle. There was a cavity in the upper lobe of the left lung, with a bronchopneumonic area in the lower lobe, and metastatic abscesses occurred in the skin. The organism was found in all of these lesions. The culture liquefied gelatine, and was practically nonpathogenic, although when injected subdurally into guinea-pigs, miliary nodules were produced on the meningeal surfaces. Berestneff considered his strain to be identical with those of Ferré and Faguet, and Sabrazés and Riviére.

Scheele and Petruschky²⁷ reported a case of fatal pleuropneumonia with secondary abscesses in the skin, in a woman aged 56. Acidfast branching filaments were found in the sputum and in pus from the dermal abscesses during life. The organism produced a firm white growth on agar, better on dextrose agar, and did not grow at all on glycerol agar or potato. In a communication to Schabad the authors reported that it slowly liquefied gelatine, and produced only slight localized abscesses in guinea-pigs.

In addition to these cases, a number have been reported in which acidfast actinomycetes have been demonstrated in lesions or exudate, but in which it was impossible to obtain cultures.

Ljubimoff, in a personal communication to Berestneff,⁴ reported a case of fatal bronchopneumonia which he observed in 1888, in which long acidfast branching filaments were found in the sputum during life. Berestneff succeeded in demonstrating their presence in the sections of the lung removed postmortem.

Flexner's²⁸ patient was a man of 70. The symptoms were those of pulmonary tuberculosis. No sputum was obtained. Necropsy revealed a caseous bronchopneumonia of both lungs, with beginning cavity formation in places and miliary pseudotuberculous peritonitis. Smears from the exudate showed acidfast branching filaments. Cultures remained sterile. A guinea-pig inoculated with the exudate died after some weeks without demonstrable lesions.

Buchholz²⁹ observed a fatal case of empyema following bronchopneumonia with lung abscess in a steel worker, 38 years old. Branching filaments were found in the exudate, but streptococci were present as well. He believed the organism was similar to *A. asteroides*.

Musser³⁰ reported a case of fatal bronchopneumonia in a man of 37. Acidfast rods and long branching filaments were found in the sputum. Cultures were unsuccessful. Guinea-pigs inoculated with sputum died from Streptococcus infections.

²⁸ Jour. Exper. Med., 1898, 3, p. 435.

²⁹ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 24, p. 470.

³⁰ Proc. Philadelphia Co. Med. Soc., 1901, 22, p. 245.

Butterfield's³¹ case was that of a young man who died of diabetic coma. He had had a cough for some time. Tubercle bacilli were not found in the sputum. At necropsy a consolidated area with a central cavity in the right upper lobe was found. Smears showed acidfast branching filaments.

Ribytkof and Maloletkof reported a case of spinal cord abscess with meningitis in which acidfast actinomycetic mycelium was demonstrated. No portal of entry for the infection was found.

Foulerton²² found acidfast mycelium in the sputum of a man 50 years old who had suffered from pulmonary disease for a year. The organism could not be cultivated and proved nonpathogenic for guinea-pigs. Foulerton also reported another case personally communicated to him, in which similar structures were found in the sputum; the patient was a man of 28, who developed pulmonary disease following the removal of what was believed to be a tuberculous kidney. The outcome of these two cases is not reported.

The case that we report is of interest because the condition was diagnosed during life; the organism was isolated in pure culture from the sputum and proved to be different from any of the recorded species; immunologic studies were made and vaccine treatment was tried.

The patient was a woman of nervous type, 31 years old, married, who had two children. She complained of cough, periodic expectoration on lying down, and general weakness. About 3 years ago, following childbirth, she took cold and began to cough. She had never been well since, having frequent attacks of tonsillitis and a chronic cough. At first there was very little sputum; what little there was "appeared greenish and shiny, with a peculiar medicated odor." But about 2 years ago she began coughing up large quantities of sputum, a cupful or more during the day. The sputum was occasionally blood streaked. She gradually developed a goiter for which an operation was performed one year ago. Three months later she began to cough more and had severe pain in the right side of the chest. There was fever for several days at this time, and for several months there was a degree of fever every day or so.

She was first seen July 18, 1919. Physical examination at this time revealed nothing noteworthy save an area of impaired resonance with crackling râles in the right axillary region from the fifth to the eighth ribs. Roentgen screen examination showed slight thickening of both apexes and small annular shadows near the hilum of the right lung. There were no areas that suggested massive consolidation or large accumulations of pus.

The laboratory findings were negative with the exception of the sputum, which will be described later. The temperature showed no abnormal variations during two weeks.

Intracutaneous tuberculin tests were positive both with Koch's O. T. and tuberculin prepared from tubercle bacilli treated with CO₂ under high pressure, according to the method of Larson,³² producing areas of infiltration the size of a half-dollar in 48 hours.

The sputum was examined for the presence of tubercle bacilli by the Ziehl-Neelson method. The examination revealed the presence of acidfast branching filaments. For the most part these were isolated strands showing lateral branches at irregular intervals, but occasionally there was found a consider-

³¹ Jour. Infect. Dis., 1905, 2, p. 421.

³² Jour. Infect. Dis., 1918, 22, p. 271.

able mass of mycelium in which the central filaments were closely intertwined, the peripheral portions being irregularly arranged in a radial fashion, resembling somewhat the sulphur granules of ordinary actinomycosis. For the most part, the filaments were quite uniform in diameter, although occasional fusiform swellings occurred; but definite clubbed extremities were never found. In addition to these structures, there were many mycelial fragments of varying length, some showing a remarkable resemblance to true tubercle bacilli. The mycelium was not uniform in its acidfastness, some strands taking the blue counterstain. Moreover, it was not so acidfast as tubercle bacilli; for, if the 5% hydrochloric acid-alcohol used as a decolorizing agent was allowed to act for a minute or more, practically all of the mycelium was decolorized. Several specimens were examined during the course of 2 weeks, and all showed the presence of this organism, although in some specimens they were much more numerous than in others. There were numerous bacteria of various kinds in the sputum and all attempts to isolate the organism by plating directly from the sputum were unsuccessful, all the plates being overgrown by spreading colonies of bacteria.

Two guinea-pigs were inoculated intraperitoneally with sputum. These died after 5 and 6 days. Necropsy revealed small tubercle-like nodules scattered over the peritoneal surface, being most numerous on the under surface of the diaphragm and the surface of the liver and spleen. The omentum was shrunk to a thick mass in the upper abdomen, studded with nodules. A few flakes of fibrin were present on the peritoneal surfaces, and the parietal peritoneum in addition showed hemorrhages.

From the peritoneal tubercles removed aseptically and crushed, the organism was readily grown in pure culture. The colonies in glycerol agar appeared in 24 hours as small white dots which under the microscope showed the star-like structure described by Eppinger. Subcultures from these colonies grew on all mediums, growth appearing over night.

On agar the growth appears first as a thin grayish veil that soon becomes thick, opaque and chalky-white in color, the surface being dry and wrinkled. The growth is firmly adherent to the medium, and when touched with the inoculating wire breaks off in large flakes, quite unlike the mealy consistence of *A. asteroides*. If the whitish bloom is scraped off, the underlying mycelium is found to have a buff color, but never assumes the distinct yellow or orange of *A. asteroides*. We have compared our strain with two subcultures of Eppinger's organism obtained from different sources.

On agar containing various carbohydrates, such as dextrose, maltose, and glycerol, growth is more rapid and more luxuriant than on plain agar, but otherwise it is quite the same. The growth on potato is similar to agar. On Dorsett's egg medium and Loeffler's blood serum, growth is not so rapid. Neither of these mediums are digested.

In gelatine, stab culture growth occurs only on the surface. The medium is liquefied, digestion beginning in a few days and being complete in a week. The liquefaction is stratiform.

In liquid mediums growth occurs only on the surface, appearing first as small white flakes floating on the surface which soon coalesce to form a thick, wrinkled, snow-white pellicle, turning up at the edges to extend a centimeter or more on the glass container. After about 2 weeks this pellicle breaks on jarring and falls to the bottom of the vessel, after which no further growth occurs.

A similar pellicle, having a somewhat yellowish tinge, is produced on litmus milk. The milk turns alkaline and after a few days is curdled, apparently due

to the secretion of rennin. At this time the litmus is reduced. The coagulated casein is then slowly digested, peptonization being complete in about 2 weeks.

None of the carbohydrates were fermented. Starch agar plates failed to show any diastatic action. Blood agar showed no hemolysis.

All of the meat extract mediums showed some change in color after a time, the mediums becoming darker and assuming a brownish tint. This change also occurred in Dunham's peptone solution, where it was more striking, the colorless fluid first assuming a straw color just beneath the pellicle, the color then gradually diffusing through the medium and after some weeks becoming quite dark.

This brownish discoloration of medium containing meat extract or peptone is quite characteristic of many saprophytic actinomycetes found in soil, and has also occurred in such cultures of the lumpy-jaw organism as we have had an opportunity to study. It has been explained by Beijerinck as being due to the formation of quinone, and is associated with the fermentation of tyrosine. Aoyama and Miyamoto observed a darkening of the mediums with their strain, but otherwise we have found no mention of this reaction with acidfast actinomycetes. It did not occur with the two subcultures of *A. asteroides*, which we examined.

Our organism grew readily on such simple mediums as Czapek's agar, containing only inorganic salts and saccharose, and agar made from an infusion of soil.

A broth culture several days old was shaken till the pellicle was pretty well broken up, and inoculated in 1 cc quantities intravenously into rabbits and intraperitoneally into guinea-pigs.

The rabbits died in about 48 hours. Necropsy revealed minute abscesses in various viscera, but especially numerous in the kidneys which were studded with minute, yellowish white dots that bulged slightly above the cortex. On section, these were found scattered throughout both cortex and medulla. Microscopic examination revealed them to be simple aggregations of polymorphonuclear leukocytes.

The guinea-pigs died in from 4 to 6 days. The lesions were exactly like those described for the guinea-pigs inoculated with sputum. Sections of the nodules showed them to be small abscesses containing necrotic material and pus cells in the center, and encapsulated by a zone of granulation tissue in which mononuclear leukocytes and proliferating fibroblasts were the only cellular elements.

After 8 months' cultivation, the virulence for animals was undiminished.

Having obtained a pure culture, we prepared an antigen for immunologic studies. The organism was grown in flasks on broth containing 3% each of dextrose and glycerol, until the pellicle broke, after about 10 days. It was then heated to 60 C. for one hour, and placed in Dr. Larson's apparatus under the pressure of a fresh tank of carbon dioxide over night. On releasing the pressure it was found that the masses of mycelium had disintegrated, forming a uniform suspension of mycelial fragments. The organism, however, still grew when subcultivated; the suspension was again heated to 65 C. for an hour, and 0.25% of tricresol added. It was then found to be sterile.

Part of this material was used in this condition, while another portion was passed through a porcelain bougie, and the clear filtrate was used.

Skin tests were made with both of these antigens, injecting 0.1 cc of a 1:1,000 dilution intracutaneously. That night the patient had a chill, the temperature rose to 102 degrees, and she ached all over. The next day the tem-

perature was normal. In spite of this severe constitutional reaction, there was no visible reaction at the point of inoculation after 48 hours.

The antigen prepared as above proved nonhemolytic and not anticomplementary, and was therefore used for complement fixation tests with the patient's serum. The reaction was found to be strongly positive, the complement being completely bound, using 2 drops of the patient's serum and 2 drops of the antigen, and the antishoop hemolytic system ordinarily used in our Wassermann tests. A similar test made with this antigen and the serums of 12 other persons gave negative results throughout.

As the mycelial fragments, after treatment with CO₂ under pressure remained in suspension for some time, the antigen was also useful for agglutination tests. The tests were made by the macroscopic method, incubating at 55 C. for an hour, and read after standing in the icebox over night. The patient's serum agglutinated completely in a 1:20 dilution, and partially at 1:100; a control serum produced no agglutination.

The antigen prepared as described above was used for vaccine treatment, beginning one week after the intracutaneous injections. The vaccine was injected subcutaneously, given at intervals of 4 or 5 days. The first three doses were 0.1 cc, 0.5 cc, and 0.5 cc of the 1:1,000 dilution; the fourth and fifth doses were 0.1 cc and 0.5 cc of the 1:100 dilution, and the sixth dose was 0.1 cc of the 1:10 dilution. After the third dose there was a very slight febrile reaction and a small lump at the point of injection; otherwise, there was no reaction to the injections of vaccine.

After the last injection she returned to her home in another state. Her physician there was furnished with a supply of the vaccine, and the injections were continued for another month. After each injection a very painful lump developed, and the vaccine was discontinued for this reason; no constitutional reactions occurred. The dosage was not further increased.

Letters received from the patient in January and October of 1920 indicate that the disease has progressed somewhat; the cough and expectoration continue, and on 3 occasions there have been hemorrhages from the lungs. Following these hemorrhages there has been fever, but at other times her temperature has been normal. She gained weight for a while and then lost 12 pounds, but has been gaining weight lately. She has complained much of pain in the chest.

Injectons of vaccine were continued; the patient was also placed on the usual tuberculosis regime, but otherwise no treatment was given. On returning to her home she again resumed her usual household work.

Infections with acidfast actinomycetes cannot be frequent, as we have been able to find but 26 reported cases. Undoubtedly they are more frequent than is indicated by these reports, however, for some cases have probably been seen which have not been published, and certainly a still greater number have been undiagnosed. The disease shows no particular geographic distribution, cases having been reported from America, England, France, Germany, South Africa, Japan, and the Philippine Islands.

The ages of the patients have varied from 4 weeks to 70 years; with the exception of 2 cases in young children, the ages are fairly

evenly distributed throughout adult life. The majority of the cases occur in males. There is considerable evidence that the actinomycoses are occupational diseases, the majority of cases occurring in men who handle grain or straw or similar material, on which the organisms are believed to live. With the acidfast actinomycetes, however, no such condition can be shown, for only one patient in whom the occupation is recorded was a handler of grain. Several patients, however, were engaged in occupations that would be likely to lead to pneumokonioses. With the exception of 3 cases, 2 of postoperative peritonitis and one of Madura foot, the infection has been primary in the pulmonary tract, although in several cases this has been assumed rather than proved. A survey of the necropsy findings indicates that the sequence is somewhat as follows: The first lesions are in the peribronchial lymph nodes, the organisms probably having been introduced by inhalation. Death may follow metastatic infection from this area without involvement of the lungs, as in Eppinger's case; but more frequently a caseous bronchopneumonia occurs followed by central softening and cavity formation. The infection may remain localized in the lungs, but frequently is accompanied by empyema or by metastases, the latter localizing most frequently in the brain. In several cases there was apparently a hematogenous distribution shortly before death with the production of numerous miliary pseudotubercles.

The duration of the illness varies from 2 weeks to 3 years, with about 6 months as the average. Some cases have been relatively acute.

Microscopically the lesions in man show necrosis with pus cells in the neighborhood of the mycelium. These areas are soon surrounded by granulation tissue containing mononuclear cells, but giant cells do not occur. The lesions in experimentally inoculated animals are quite similar; it is only when strains of attenuated virulence, or resistant animals are used that lesions histologically resembling true tubercles are found. The histogenesis of the lesions in animals has been extensively studied by MacCallum² and by Nakayama.¹

Less than half of the cases have been diagnosed antemortem. The disease is naturally confused with tuberculosis. The diagnosis is established by finding long branching acidfast filaments in the sputum. Frequently a great deal of the mycelium has undergone fragmentation, and bacillary forms may be found in many fields before intact mycelium is seen. At first glance these mycelial fragments resemble tubercle bacilli quite closely, and may lead to erroneous diagnosis. Birt and Leish-

mann²³ report that they were thus led astray on their first examination of the sputum from their case, and Foulerton believes that many cases have been diagnosed as tuberculosis for this reason. The mycelial fragments in our case, however, showed much greater variation in length than is common with tubercle bacilli.

Zupnik observed that guinea-pigs inoculated with acid-fast actinomycetes gave positive tuberculin reactions, and our patient gave markedly positive von Pirquet tests. We unfortunately failed to test our patient's serum against tubercle bacillus antigens for complement fixation, and also have neglected to test the serums of tuberculous patients against our antigen, so that we are unable to state whether or not complement-fixation tests can be of use in diagnosis. Claypole,³³ however, has observed that guinea-pigs inoculated with acidfast actinomycetes bound complement with tubercle bacillus antigens, and vice versa. It is clear, therefore, that from the standpoint of immunity tests these organisms are very closely related, and if serum reactions are to be used in differential diagnosis, they must be made quantitative.

The prognosis is not good. The case of Madura foot recovered following amputation; in several cases the outcome was not reported; the remainder terminated fatally.

There is nothing in the collected literature to guide us in the matter of treatment. Iodides, which have proved successful in some cases due to other actinomycetes, have apparently not been used in any infections due to the acidfast group. In view of the disastrous results that have been reported following the use of the iodides in pulmonary tuberculosis, it is doubtful that their administration in these infections would be justified. Wynne has reported the successful use of an autogenous vaccine in a case of pulmonary actinomycosis due to a nonacidfast variety. We are unable to state whether or not our patient was benefited by the vaccine. The fact that she was alive a year after the treatment was begun cannot be overlooked when one considers the progressive downward course of the cases hitherto reported.

In summarizing the bacteriologic data that have been published, it is clear that at least three distinct species from human sources have been previously described, ours making a fourth.

A. asteroides (Eppinger) is characterized by a growth of mealy consistence on solid medium, varying from pale yellow to deep orange in color. It does not liquefy gelatine or peptonize milk and is more

³³ Jour. Exper. Med., 1, 1913, 17, p. 99.

pathogenic for guinea-pigs than for rabbits. It has been reported more frequently than the other varieties.

The strain isolated by Aoyama and Miyamoto apparently occupies a position midway between Eppinger's variety and the next species. It differs from *A. asteroides* only in the whitish color of the growth and the darkening of the medium. It apparently resembles somewhat *A. caprae*. Bernstein's strain was apparently similar to this one.

The unnamed species described by Birt and Leishman is characterized by a snow-white growth on solid medium. It does not liquefy gelatine but does peptonize milk. It is pathogenic for guinea-pigs; rabbits were not inoculated.

The Berestneff type is characterized by a gray to whitish growth and the liquefaction of gelatine. It is nonpathogenic for laboratory animals.

Our variety resembles the strains of Aoyama and Miyamoto and of Birt and Leishman in the white appearance of the growth on solid media. This whiteness is due to the aerial spores which develop very early; we would characterize it as a chalky white, since the surface is without luster. Our organism is apparently more virulent for laboratory animals than those hitherto reported from man, in this respect resembling most closely the strain isolated by Zimmerman from the guinea-pig; rabbits are distinctly more susceptible than guinea-pigs.

Our strain is particularly characterized, however, by its proteolytic activity, as evidenced by the alkaline coagulation of milk (rennin), the digestion of the coagulated casein, the rapid liquefaction of gelatine, and the darkening of mediums containing peptone (tyrosinase). In this respect it resembles the organism of lumpy-jaw and the saprophytic soil actinomycetes. In fact, we can arrange the various species of acidfast actinomycetes in a fairly uniform series, with Eppinger's variety at one extremity establishing contact with the acidfast bacteria, which it resembles very closely in cultural characters, and our own strain at the opposite extremity, where it joins the nonacidfast actinomycetes. Because of the chalky-white appearance of the growth on mediums, we propose for our new species the name *Actinomyces gypsoides*.

THE GROWTH OF THE GONOCOCCUS IN VARIOUS GASEOUS ENVIRONMENTS

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That micro-organisms are sensitive to respiratory gases has been discussed for a number of years. Pasteur showed that, in the free access of oxygen, the yeast plant fermented the sugars very slowly; while if grown in the presence of a large excess of CO₂, fermentation proceeded at its maximum pace. This alone shows the effect of respiratory gases on fermentation.

C. Fränkel¹ showed that some bacteria, namely, *B. typhosus* and Friedländer's bacillus, grow as well under pure CO₂ as in atmospheric conditions; other strains, such as *B. prodigiosus*, *B. proteus* and *B. phosphorescens* will grow under pure CO₂ but not as luxuriantly as under aerobic conditions; while still a third group will not grow at all under pure CO₂ namely, the spirillum of Asiatic cholera, *B. anthracis*, *Micrococcus tetragenus*, the bacteria of chicken cholera and of rabbit and mouse septicemia. Of this last group, all will grow in a mixture of CO₂ and air, or some will grow under pure CO₂, if the temperature of incubation is raised. He also says that the same holds true in a general way in atmospheres of pure oxygen.

Ferran² and Belfonti³ found that the tetanus bacillus could be successfully habituated to aerobic conditions by the gradual increase of oxygen in cultures. We know that as a rule most anaerobes are sensitive to exposure to oxygen, yet here is a case in which an anaerobic bacterium could be transformed into an aerobic one.

Wherry and Oliver⁴ claimed that the gonococcus when first isolated grows best under a partial tension produced by the hay bacillus.

Wherry and Ervin⁵ showed that the removal of CO₂ inhibited the growth of a recently isolated tubercle bacillus.

Cohen and Markle,⁶ Cohen,⁷ and Cohen and Fleming⁸ published several articles on the advantage of using a reduced oxygen tension and an increased CO₂ tension in the isolation of the meningococcus.

Chapin⁹ and Kohman¹⁰ also reported on the advantage of CO₂ in the growth of the gonococcus and the meningococcus, but St. John¹¹ claimed that the

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¹ Ztschr. f. Hyg. u. Infectiouskr., 1889, 5, p. 332.

² Centralbl. f. Bakteriöl., 1898, 24, p. 28.

³ Arch. per le. sc. med., 16, cited by Hiss and Zinsser.

⁴ J. Infect. Dis., 1916, 19, p. 288.

⁵ Ibid., 1918, 22, p. 194.

⁶ Jour. Am. Med. Assn., 1916, 67, p. 1302.

⁷ Ibid., 1918, 70, p. 1999.

⁸ Jour. Infect. Dis., 1918, 23, p. 337.

⁹ Ibid., 1918, 23, p. 342.

¹⁰ Jour. Bacteriol., 1919, 4, p. 571.

¹¹ Med. Rec., 1919, 95, p. 184.

respiratory gases were not an important factor in the growth of the meningococcus; stating, however, that the advantage of the method as suggested by Wherry and Oliver was due to increase in moisture from sealing the tube.

Gates¹² claimed that the advantage of Cohen's method for the isolation of the meningococcus was due to the increased moisture content obtained by sealing the tube. Gates and Kohman thought that the effect of CO₂ on the growth was not due to direct influence on respiration but to the action of the CO₂ on the medium tending to acidify alkaline medium, thus providing a delicate means of regulating the H-ion concentration.

Lately Swartz and Davis¹³ described what they believe to be a simpler method of producing partial tension (by heating the tube) than the method involving the use of the hay bacillus. Further, it seems from their work that partial tension is a more satisfactory method for isolation of the gonococcus than the aerobic method.

In view of the conflicting ideas held by various workers as to the relative value of the influence of the gaseous environment, moisture and reaction on the primary cultivation of the gonococcus, we decided to try to analyze the effect of these factors.

Technic.—In these experiments two strains were used. Strain A, from a case of gonorrheal ophthalmia, was isolated on ascites agar under partial tension with the hay bacillus (fig. 1). Strain B was isolated in the same way from a case of vulvovaginitis.

The medium used with culture A was NaCl ascites agar with a P_H 7.4; and with culture B, Na₂HPO₄ ascites agar with a P_H 7±. The agar was made with beef infusion broth containing 1% of peptone (Difco) and 0.5% salt or sodium phosphate. The ascites fluid was added in the proportion of one part to four of the tubed and sterilized agar.

The gases used were obtained in steel cylinders. A mixing chamber, as shown in fig. 2, was devised for procuring the proper mixture of the gases. The culture tubes used were of large size, fitted with two hole rubber stoppers, through which a short and a long glass tube were passed as shown in fig. 3. The free ends of the glass tubes were fitted with gum rubber tubing and screw clamps. All connections were sealed with a mixture of paraffin and Canada balsam (see bottom of illustrations).

1. *Experiments.*—The stock cultures grown since the time of isolation under partial tension (hay bacillus) were transplanted to various gaseous environments to determine whether growth would occur under such sudden environmental changes. The results were:

¹² Jour. Exper. Med., 1919, 29, p. 321.

¹³ Jour. Am. Med. Assn., 1920, 75, p. 1124.

It will be noted that when derived from a partial tension culture, the gonococcus grew best under similar conditions or under pure hydrogen, and that growth did not occur under high concentrations of CO_2 or O_2 ; however, as will be shown later, it can be adapted to do so.

TABLE 1
RESULT OF TRANSPLANTATION OF STOCK CULTURES

Series A Source, From Partial Tension		Series B Source, From Partial Tension		
Gas Environment	Growth in 24 Hours	Gas Environment	Growth in 24 Hours	Growth in 48 Hours
100% H_2	+	Aerobic.....	+	+
100% CO_2	—	Partial tension.....	+++	+++
50% H_2 , 50% CO_2	—	Anaerobic (Wright's).....	—	—
75% H_2 , 25% CO_2	±	100% H_2	+	++
100% O_2	—	CO_2 100%.....	—	—
50% O_2 , 50% N_2	—	O_2 100%.....	—	—
50% O_2 , 50% H_2	—			

— No growth
± Just perceptible growth
+ Delicate growth

++ Fair growth
+++ Good growth
++++ Very luxuriant growth

2. Adaptation to Hydrogen and the Tenacity of this Adaptation.—

1. It was noted that the gonococcus would grow readily under H_2 directly from partial tension (table 1). This obviated the necessity of slowly adapting the organism to this gas. Transplants were made daily and maintained under hydrogen for periods varying from $1\frac{1}{2}$ to 3 months before the following experiments were made (table 2).

TABLE 2
EXPERIMENTS WITH TRANSPLANTS FROM H_2

Series A				Series B			
Gas Mixtures	Hours			Gas Mixtures	Hours		
	24	48	72		24	48	72
Air (sealed tube).....	—	+	++	Air (not sealed).....	—	±	++
100% CO_2	—	—	—	100% CO_2	—	—	—
60% air, 20% H_2 , 20% CO_2	—	±	++	100% O_2	—	—	—
Anaerobic (Wright's).....	—	—	—	Anaerobic (Wright's)	—	—	—
100% H_2	+++	+++	+++	Anaerobic (Wright's)	—	—	—
				dextrose medium....	—	—	—
				Partial tension.....	++	++	++

It will be noted that such a strain adapted to grow under H_2 had its growth inhibited by the presence of O_2 or CO_2 and grew best under H_2 or the partial tension method. When O_2 was removed from the air by pyrogalllic acid and caustic soda no growth occurred.

3. *Adaptation to Oxygen.*—In the following successive subcultures the amount of O_2 in the air was increased; each transplant representing a subculture from the preceding one.

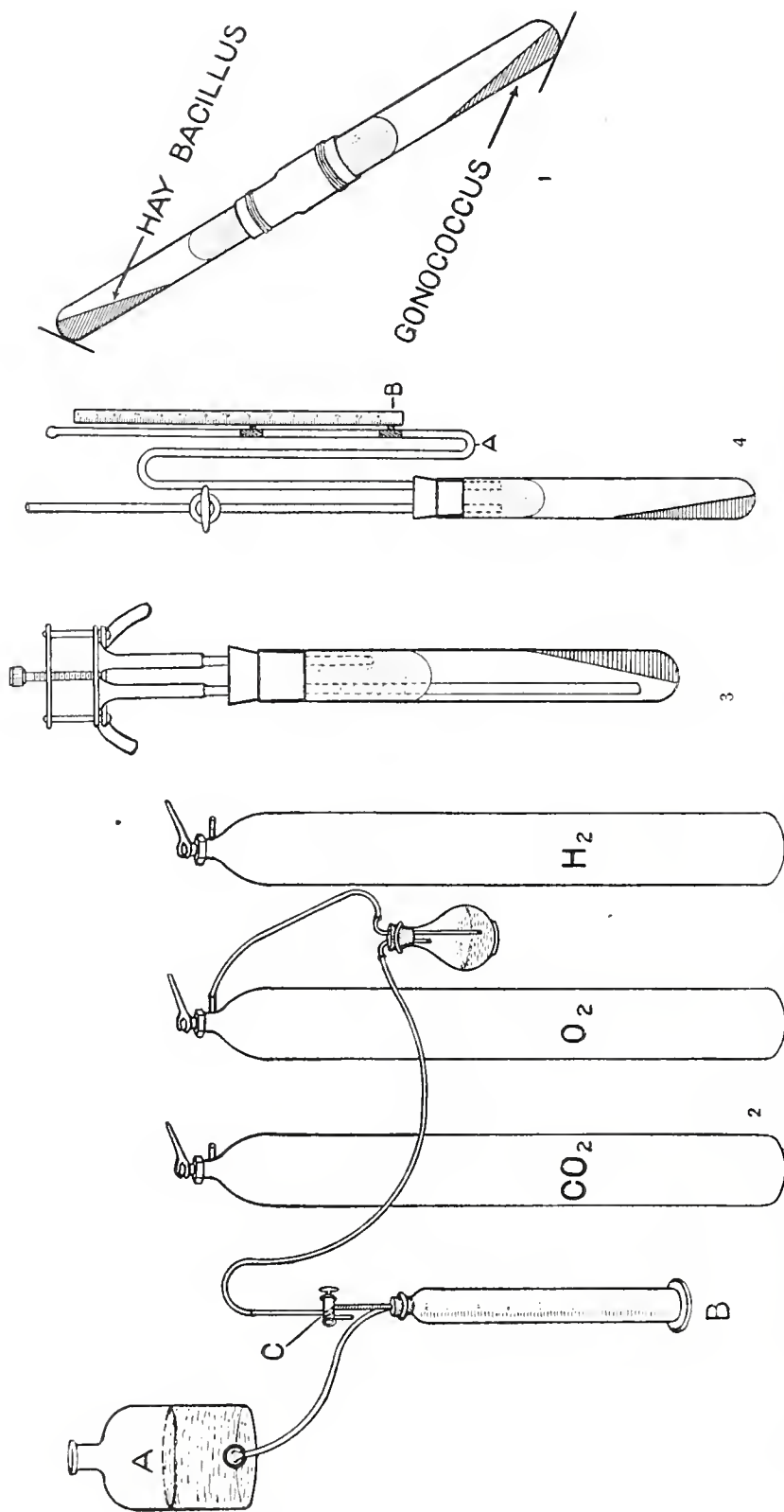


Fig. 1.—Apparatus used to isolate strain A from a case of gonorrheal ophthalmia isolated on ascites agar under partial tension with hay bacillus. Fig. 2.—The apparatus used in producing the various gas mixtures. The gases used were obtained in steel cylinders as illustrated. From these cylinders they were passed first to the wash bottle, which contained water, and merely indicated the rate of flow of the gases. From there the tube led to the graduated cylinder B, through a two way stopcock C, which permitted all air to be driven from the tubes before the gases were admitted to the mixing chamber (B), which was filled with water. Gases were admitted to B through a short glass tube and were measured by displacement of water to the reservoir A through a long glass tube reaching from the bottom of the cylinder B to the rubber tube connecting the reservoir. The water level in the reservoir was 4 feet above the base of the mixing chamber when no gases were in the cylinder, and only 2 inches higher when all the water in the cylinder had been displaced. Therefore the variations in the pressure on the gas volumes amounted only to approximately the height of the cylinder B. This was corrected in the graduation of the cylinder. The water in the system was made acid to prevent absorption of CO_2 when that gas was used.

After the desired percentages of gases had been introduced into the chamber and mixed, the tube from the tanks was withdrawn and a short, curved glass tube was attached to the top of the cylinder B, the inlet now becoming the outlet.

Fig. 3.—The culture tube. The long arm was thoroughly flamed and slipped past the cotton plug. The short arm did not penetrate the plug which was packed tightly around the long arm. To the short arm was attached the tube from the mixing chamber and the gas mixture under pressure from the reservoir was allowed to displace the air in the culture tube. Regardless of the specific gravity of the gas, it was always introduced through the short arm, being thus filtered through the cotton plug. The plane of the culture tube was altered depending on the specific gravity of the gas mixture. The air was allowed to escape through the long arm. If gases were introduced through the long arm, and so unfiltered through the cotton, contamination invariably resulted. Many times the amount of gas necessary to fill the tube was passed through, to avoid as much as possible the error of allowing air to remain in the cotton plug or elsewhere, altering the gas concentrations.

Fig. 4.—Apparatus used in Exper. 6.—All connections were sealed with Canada balsam and paraffin. The tube with the glass stopcock was attached to a vacuum pump and air removed until the manometer A recorded several mm. less than atmospheric pressure. The stopcock was closed and the tube incubated in this condition.

In series A the transplants were made from the earliest appearing colonies. In series B the subcultures were made from colonies appearing in 24-48 hours. Transplant 8 (Series B) was carried to O₂ without air several times. The growth was in all instances delayed, and subcultures failed after the first or second transplants.

Summarizing table 3, we may conclude that the gonococcus may be adapted to growth in concentrations of O₂ above those which previously inhibited its growth.

TABLE 3
RESULTS OF TRANSPLANTATION

Series A First Transplant from Aerobic Culture				Series B First Transplant from Partial Tension		
Gas Mixtures	Hours			Gas Mixtures	Hours	
	24	48	60		24	48
1. 20% O ₂ , 80% air.....	++	1. 25% O ₂ , 75% air....	++	+++
2. 50% O ₂ , 50% air.....	—	±	++	2. 25% O ₂ , 75% air....	++	+++
3. 50% O ₂ , 50% air.....	—	±	++	3. 35% O ₂ , 65% air....	++	++
4. 50% O ₂ , 50% air.....	±	+	++	4. 50% O ₂ , 50% air....	+	++
5. 50% O ₂ , 50% air.....	+	+	..	5. 65% O ₂ , 35% air....	+	++
6. 65% O ₂ , 35% air.....	±	+	++	6. 75% O ₂ , 25% air....	+	++
7. 100% O ₂	±	+	++	7. 80% O ₂ , 20% air....	±	++
8. 100% O ₂	±	+	++	8. 86% O ₂ , 14% air....	±	++
9. 100% O ₂	±	+	++	9. 100% O ₂	±	+
				10. 100% O ₂	—	—

TABLE 4
TENACITY OF ADAPTATION TO OXYGEN

Series A All Transplants from 100% Oxygen				Series B All Transplants from 80% O ₂ , 20% Air			
Gas Mixtures	Hours			Gas Mixtures	Hours		
	24	48	72		24	48	72
Air.....	++	++	..	100% CO ₂	—	—	..
100% H ₂	—	—	—	Air.....	+	++	..
100% CO ₂	—	—	—	100% H ₂	—	±	bubbles in me- dium
75% H ₂ , 25% CO ₂	—	—	—				

Having determined that such an adaptation to high concentration to O₂ could be brought about, the following experiments were made in order to study the respiratory requirements of such an adapted strain (table 4).

It is evident from comparison of table 4 with the preliminary experiment (table 1) that an O₂ adapted strain grows more readily under aerobic conditions than an unadapted culture, especially one from partial tension. Further experiments showed that such an aerobic sub-

culture could be transplanted back to the high O_2 concentration providing that the culture in the first instance had been kept under O_2 for a sufficient length of time.

The action toward other gaseous environments is unchanged except that it seems to grow less under hydrogen.

4. *Adaptation to CO_2 .*—As shown in table 2, the gonococcus grown aerobically cannot be transferred suddenly to a high concentration of CO_2 ($4 CO_2 + 1$ air) but the experiments in table 5 show that such an adaptation can be gradually brought about. One strain was maintained in 48-hour subcultures in almost pure CO_2 for two months. The transplants are derived each from the preceding one:

TABLE 5
ADAPTATION OF TRANSPLANTS TO CO_2

Series A First Transplant from Aerobic Culture			Series B First Transplant from Partial Tension			
Trans- plant	Gas Mixture	24 Hours	Trans- plant	Gas Mixture	24 Hours	48 Hours
1	CO_2 14%, air 86%.....	++	1	CO_2 25%, air 75%.....	+	++
2	CO_2 18%, air 82%.....	++	2	CO_2 40%, air 60%.....	+	++
3	CO_2 30%, air 70%.....	++	3	CO_2 50%, air 50%.....	+	+
4a	CO_2 86%, air 14%.....	—	4	CO_2 50%, air 50%.....	++	..
4b	CO_2 30%, air 70%.....	++	5	CO_2 60%, air 40%.....	++	..
5	CO_2 37%, air 63%.....	++	6	CO_2 66%, air 34%.....	++	..
6	CO_2 37%, air 63%.....	++	7	CO_2 75%, air 25%.....	++	..
7	CO_2 43%, air 57%.....	++	8	CO_2 80%, air 20%.....	++	..
8	CO_2 50%, air 50%.....	++	9	CO_2 86%, air 14%.....	++	..
9	CO_2 50%, air 50%.....	++	10	CO_2 100%.....	++	..
10	CO_2 66%, air 34%.....	++	11	CO_2 100%.....	±	+
11	CO_2 75%, air 25%.....	++	12	CO_2 100%.....	++	..
12a	CO_2 100%.....	—				
12b	CO_2 80%, air 20%.....	—				
12c	CO_2 75%, air 25%.....	++				

Tenacity of Adaptation to CO_2 .—When adapted to high concentrations of CO_2 the strain is unfavorably affected by O_2 but still retains its ability to grow under partial tension and H_2 . In a few instances in which growth occurred under aerobic conditions, subcultures were not viable (table 6).

TABLE 6
EQUALITY OF ADAPTATION TO HIGH CONCENTRATIONS OF CO_2

Series A All Transplants from CO_2 /Air = 3/1				Series B All Transplants from CO_2 ("pure")		
Gas Mixtures	Hours			Gas Mixtures	Hours	
	24	48	60		24	48
100% H_2	—	±	+	100% H_2	—	+
20% air, 80% H_2	±	+	++	100% O_2	—	—
Aerobic.....	—	—	—	Partial tension.....	++	++
Air (sealed tube).....	—	±	++	Air (open tube).....	—	—

5. *Factors Concerned in Delayed Growth.*—It will be noted that in the experiments made to test the tenacity of the adaptation of the carbon dioxid and hydrogen strains, in certain cases there was delayed growth of the subcultures (tables 2 and 5). The following set of experiments was devised to study the factors producing this delayed growth (table 7).

TABLE 7
EXPERIMENTS MADE TO SHOW FACTORS PRODUCING DELAYED GROWTH

Series A All Transplants from CO ₂ /Air = 3/1			Series A All Transplants from 100% H ₂			
Gas Mixtures	Result in Hours		Gas Mixtures	Result in Hours		
	24	48		24	48	72
50% CO ₂ , 50% O ₂	—	+	CO ₂ 50%, O ₂ 50%.....	—	±	..
66% CO ₂ , 34% O ₂	±	+	H ₂ 50%, CO ₂ 50%.....	±	±	..
50% CO ₂ , 50% H ₂	+	+	H ₂ 66%, CO ₂ 34%.....	+	+	..
50% O ₂ , 50% H ₂	—	—	H ₂ 25%, O ₂ 75%.....	—	—	+
			H ₂ 50%, O ₂ 50%.....	—	±	..
			H ₂ 66%, O ₂ 34%.....	—	+	..
			H ₂ 75%, O ₂ 25%.....	—	++	..

From study of table 7 on strains adapted to CO₂ and H₂, it appears that oxygen seems to be an important factor in producing delayed growth.

6. *Study of the Enclosed Gases.*—The next question presenting itself concerned quantitative changes in the enclosed gases due to respiration of the organisms. In order to determine whether gas is produced by the organisms, they were transplanted into a closed tube connected with a mercury manometer shown in fig. 4. A pressure of several mm. less than the atmospheric was produced by a vacuum pump and the cultures incubated. The results are shown in table 8.

TABLE 8
RESULTS OF TRANSPLANTATION OF ORGANISMS INTO CLOSED TUBE CONNECTED WITH
MERCURY MANOMETER

Series A Transplant from H ₂ Environment for 3 Months			Series B Transplant from Partial Tension		
Time in Hours	Reading 37 Degrees	Growth	Time in Hours	Reading 37 Degrees	Growth
0	83 Mm.	—	0	90 Mm.	—
14	66 Mm.	—	24	60 Mm.	—
24	41 Mm.	±	36	33 Mm.	±
30	25 Mm.	+	48	0 Mm.	++
42	0 Mm.	++			

The same apparatus connected to an uninoculated tube of sterile medium was incubated as a control for 3 days, and no changes in pressure occurred. From table 8 it seems that bacteria respire before growth appears and growth is not visible until considerable change in the gaseous environment has occurred.

An attempt was made to determine the nature of these gaseous changes. An analysis of the gas from a 48-hour aerobic subculture grown under air, sealed with a rubber stopper and fitted with double glass stopcocks was made, with the following results: Culture CO_2 , 6%, O_2 2.5%; control (laboratory air) CO_2 0.5%, O_2 19%. Residual gas; noncombustible in either case.

An analysis of gas from a 30-hour growth under hydrogen, transplanted from a H_2 adapted strain showed: Culture: CO_2 3%, O_2 0.2%; control (tube filled with H_2) CO_2 0%, O_2 0.6%. Remainder combustible in both cases.

It seems that O_2 certainly is used by the gonococcus and CO_2 given off in aerobic and H_2 cultures.

7. *The Effect of Moisture Compared with that of Gases.*—Method: Four transplants were made: aerobic; to its adapted gas requirement; under its gas requirement with CaCl_2 in the neck of the tube; aerobic in desiccator containing water, the desiccator being opened to air frequently. The purpose of this series of transplants was to put the organism under its previous gas requirement with and without moisture; and ignoring the gas requirement—with moisture and without moisture (table 9).

TABLE 9
RESULTS OF EXPERIMENTS ON FOUR TRANSPLANTS CONCERNING EFFECT OF MOISTURE
WITH EFFECT OF GASES

Previous Gas History		Gaseous Environment, Etc.	Result in Hours	
			24	48
Series A:				
1	Adapted to 75% CO ₂	Aerobic.....	—	..
2	Adapted to 75% CO ₂	75% CO ₂	+++	..
3	Adapted to 75% CO ₂	75% CO ₂ + CaCl ₂	++	..
4	Adapted to 75% CO ₂	Aerobic + water in desiccator.....	—	..
1	Adapted to 98% H ₂	Aerobic.....	—	..
2	Adapted to 98% H ₂	98% H ₂	+++	..
3	Adapted to 98% H ₂	98% H ₂ + CaCl ₂	++	..
4	Adapted to 98% H ₂	Aerobic + water in desiccator.....	±	..
Series B:				
1	Partial tension.....	Aerobic.....	+	+
2	Partial tension.....	Partial tension.....	+++	+++
3	Partial tension.....	Partial tension + CaCl ₂	++	++
4	Partial tension.....	Aerobic + water in desiccator.....	+	+

A repetition of these experiments produced similar results. Although it is plain that moisture plays some rôle, it is also evident that the gases play an important part.

8. *Study of Viability under Gases.*—(a) Nov. 30, 1919, a culture of the gonococcus was transplanted to ascites agar and incubated under H_2 until Dec. 14, 1919, at which time a subculture from it grew, but on Dec. 22 a transplant from the latter growth showed no viability.

(b) Following this experiment repeated tests were made to determine viability under hydrogen. A typical instance is reproduced in the following data. The strain used for this experiment had been transplanted frequently under H_2 for a month. Of 5 transplants on ascites agar, incubated under H_2 for 48 hours, then allowed to remain at room temperature for 3 days, one was subcultured daily for viability with the following results (table 10).

TABLE 10
RESULTS OF SUBCULTURING TRANSPLANT FOR VIABILITY

Subculture From	Growth in Hours		Viability
	24	48	
Tube 1.....	++	++	5 days
Tube 2.....	++	++	6 days
Tube 3.....	+	++	7 days
Tubs 4.....	±	+	8 days
Tube 5.....	±	±	9 days

These results would indicate that viability under hydrogen is dependable up to one week. Dr. J. Hermanies (personal communication), in maintaining a large number of gonococcus strains, has shown that viability is increased under partial tension as opposed to aerobic conditions. The increased viability under these conditions may be due to decrease in oxygen.

SUMMARY AND CONCLUSIONS

Cultures of the gonococcus isolated at partial tension by the aid of the hay bacillus grew under pure H_2 but not under CO_2 or O_2 . When grown for some weeks under pure H_2 they seemed to become more sensitive to the deleterious influence of O_2 and CO_2 , but still grew well by the partial tension method (hay bacillus). Although growth occurred under almost pure H_2 , it failed when anaerobic conditions were produced by means of caustic soda and pyrogallie acid.

While strains isolated at partial tension will not grow under pure O_2 or pure CO_2 , they can be adapted to do so.

It was noted during these adaptation experiments that the strains were able to adapt themselves to a new gaseous environment more rapidly than they lost old adaptations; e. g., although a strain could be adapted to grow under CO_2 , it required a prolonged series of subcultures under CO_2 to make it lose its ability to grow in the presence of O_2 . A study of the delayed growth appearing in certain gas mixtures showed that an excessive supply of O_2 probably played an important part in producing the inhibition. This is supported by an analysis of the enclosed gases in a sealed culture, showing that growth begins after a certain amount of O_2 is consumed and CO_2 given off.

While moisture must play an important part, the experiments show that according to the previous gaseous adaptation of the micro-organisms their growth will be favorably or unfavorably influenced by various gas mixtures.

All cultures, regardless of their adaptation, would grow under partial tension as furnished by the hay bacillus and those adapted to H_2 or CO_2 would grow better at partial tension than under aerobic conditions.

In the continuous cultivation of the gonococcus under the various gases the viability was greatest under H_2 and least under O_2 or aerobic conditions.

As shown by Wherry and Oliver, Wherry and Ervin, Cohen and Markle and others, certain strains of the gonococcus and meningococcus grow best at partial tension when first isolated from the body; and these experiments on adaptation emphasize the importance of including the partial tension method of cultivation in the routine isolation of parasitic bacteria.

We believe that the well recognized fact that bacteria encountered in Nature are aerobic, partial tension, anaerobic, or facultative in their respiratory requirements, is an expression of their previous gaseous adaptation.

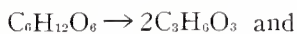
THE ACTIVITY OF STAPHYLOCOCCI IN MILK

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Staphylococci, particularly the aureus and albus types, have been isolated from a great variety of sources since they were first grown in pure culture by Rosenbach.¹ Many times several varieties have been isolated from the same source, thus causing much confusion as to their functional activities. In a study of their physiologic and biologic activities we have been considerably impressed with the various changes which they produce in milk, and consequently have made detailed studies relating to them.

Thorough investigation of bacteria includes specific studies of their morphologic, metabolic, and biologic variations, and no classification which omits one of these important factors is complete. Buchner² proved that fermentation processes, such as alcoholic, lactic, and acetic fermentations are processes not caused directly by bacteria themselves but by the enzymes they produce. Emil Fischer³ claimed that there must be a similarity between the structure of enzymes and the structure which they decompose, and he compares their resemblance in structure to that necessary between a lock and a key in order that the latter may pass the lock. Pasteur⁴ believed that fermentation corresponds to the respiration processes of animals, except that it is respiration without oxygen. Harden⁵ believed that certain organisms bring about curdling of milk by changing the sugar content to lactic acid as $C_6H_{12}O_6 \rightarrow 2CH_3CH(OH)COOH$. He thought that different strains of organisms are distinguished by a proportion of different by-products. Savage,⁶ working with *B. coli*, found that they converted soluble caseinogen to an insoluble casein but played no part in the initial curdling of milk. He ascribed this change to the action of an enzyme which these organisms produced. Kamm⁷ has recently shown that phosphates are the essential agent for favoring gas production by bacteria, while a scant supply of minerals causes bacteria to produce lactic acid. One must therefore assume that two chemical processes are carried on as



It is conceivable that the processes mentioned above and many other chemical processes may be carried out by the various bacteria functioning in different environments. Kendall⁸ defines enzymes as substances of unknown composition produced by living cells and divides them into two classes, exo-enzymes and endo-enzymes. He states that they incite specific chemical reactions and work uneconomically because they produce more utilizable

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¹ Mikroorganismen bei den Wundinfektionskrankheiten des Menschen, 1884.

² Chem. Ber., 1897.

³ Holleman's Organic Chem., 1915, p. 287.

⁴ Marshall's Microbiology, 1917, p. 156.

⁵ Proc. of the Chem. Soc., 1903, 19, p. 48.

⁶ J. Path. and Bact., 1904, 10, p. 10.

⁷ Marshall's Microbiology, 1917, p. 156.

⁸ Bacteriology, General Pathological, Intestinal, 1916, p. 49.

material for the organisms than the latter require. Little is known of the nature of enzymes but Mathews⁹ believes that they are probably colloidal protein substances.

Coagulation of milk may be brought about by two different processes, namely, addition of excess acid and action of enzyme-rennet. A question of no little importance is whether coagulation of milk by staphylococci is effected by the production of acid or by the action of enzymes. Van Slyke and Bosworth¹⁰ have studied the various changes that take place in milk due to acid coagulation. Normal cow's milk is slightly acid, having a P_H of about 6.2-6.4. According to a number of investigators, particularly Van Slyke and Bosworth, and Conn,¹¹ milk curdles when its P_H is changed to 5.4. Repeated trials using lactic acid have verified these results in this laboratory. When a sufficient amount of acid is added to milk the calcium is removed from the calcium caseinate leaving an insoluble casein in the form of a curd in the milk serum. The curd formation by the action of an enzyme in milk has been studied by Van Slyke and Publow.¹² According to these investigators, rennin first changes casein to paracasein and renders the calcium salts available for the precipitation of paracasein as calcium paracaseinate. The enzyme contained in rennet is not responsible for the coagulation of milk, but merely prepares it for chemical combinations. Curd formation is hastened by the action of excess calcium salts plus heat according to Simon.¹³ Van Slyke and Publow's report shows that in the coagulation of milk the change of casein into paracasein by an enzyme consists in a hydrolytic splitting of the casein molecule, which has a molecular weight of 8888, into two equal molecules, each with a molecular weight of 4444. Heineman,¹⁴ studying the cultural activities of hay bacilli and staphylococci has also determined that the curd is made up of an insoluble calcium paracaseinate.

My study of the enzymatic action of staphylococci in milk was conducted by attacking the problem from five different standpoints. I attempted to ascertain:

1. The amount of lactic acid necessary to form a curd.
2. The action of rennin on milk.
3. The end reaction of staphylococci on milk.
4. The daily P_H changes brought about by eight representative cultures.
5. The determination of the presence of enzymatic activity.

The experiments were repeated at three different intervals to insure constancy of results. Both desiccated and normal skim milk were tried with similar results.

⁹ Textbook of Physiological Chemistry, p. 330.

¹⁰ "Studies Relating to the Chemistry of Milk and Casein," N. Y. Exper. Station Tech. Bull. 37, 1914; N. Y. Exp. Sta. Tech. Bull. 48, "Chemical Changes in the Souring of Milk," 1916.

¹¹ Tanner's Bacteriology and Mycology of Foods, p. 114.

¹² The Science and Practice of Cheese Making, 1910.

¹³ Text Physiological Chemistry, 1917.

¹⁴ "Milk," 1919.

1. *The Action of Lactic Acid in Curdling Milk.*—Tubes containing lactic acid determined which would cause a complete curd to form.

TABLE 1
AMOUNT OF LACTIC ACID CAUSING CURD TO FORM ON MILK

Quantity	Normal P_H	Amount of Lactic Acid Added	Curd	P_H
15 c c	6.4	Neutral	None	6.4
15 c c	6.4	0.1	None	6.0
15 c c	6.4	0.2	Big curd	5.8
15 c c	6.4	0.3	Partial	5.6
15 c c	6.4	0.4	Complete	5.4

The result shows the P_H at which a complete curd is formed to be 5.4. The method used was to centrifugalize the curd, throw it to the bottom of the tube and then test the supernatant whey.

2. *Action of Rennet on Milk.*—Milk treated in the following manner with liquid rennet made by Jno. Wyeth and Bro. To tubes of milk containing 15 c c, varying amounts of rennet were added and the mixture kept at 37.5 C. Three-tenths c c of the liquid rennet were found to be the least that would give a solid curd in 24 hours. Curd formation could be produced immediately by heating over a flame. In either case there was no change in the P_H .

3. *The End Result of Growing Staphylococci in Milk.*—One hundred and eighty cultures of staphylococci, consisting of 8 *St. citreus* and the remainder about equally divided between the *aureus* and *albus* types, were used in this work. No attempt was made to subdivide either of the *aureus* or *albus* types on a basis of variation in the quantity of pigment production, although a difference of intensity of pigment was observed, particularly in the *St. aureus* group. These cultures were isolated from a wide variety of pathogenic sources, both human and veterinary. They were supplied by Cutter's Biologic Laboratories, the Mulford Laboratories, and from various hospitals about the San Francisco bay region. Repeated cultivation in milk, with brom-cresol-purple used as an indicator, gave 5 different variations. The following varieties were observed:

1. No curd formation with no P_H change.
2. Curd formation with a slight decrease in P_H .
3. No curd formation with a slight P_H decrease.
4. Curd formation with a maximum P_H decrease.
5. Curd formation plus curd digestion with increase in P_H .

The curds of the second and fourth cases when first formed appeared to occupy all the space primarily occupied by the milk, but subsequently broke away from the walls of the tube as if they were liquefying. It was found, however, that by centrifugalizing them there was no apparent change in actual quantity but that the colloidal mass of precipitated casein simply contracted down and squeezed out the whey. The majority of both aureus and albus cultures, as will be observed, belong to the third and fourth types. Table 2 shows the results of 152 cultures of staphylococci when grown in milk.

TABLE 2
RESULTS OF GROWING STAPHYLOCOCCI IN MILK

Action on Milk	Staph. aureus	Staph. albus	Staph. citreus
1. No change in milk.....	4	2	3
2. Curd formation plus slight P_H decrease.....	8	16	0
3. No curd formation plus slight P_H decrease.....	7	15	1
4. Curd formation plus minimum P_H decrease.....	45	46	3
5. Curd formation and digestion plus P_H increase....	8	2	0

4. *Tests with Eight Representative Cultures.*—The results given above indicate that various metabolic functions are performed by different types of staphylococci. Daily P_H readings, continuing through a period of 4 weeks, were carried out in an attempt to interpret the functional changes of these organisms in a special environment. A sufficient number of tubes of sterilized milk, each containing 15 c.c., was inoculated with 0.1 c.c. of a 24-hour broth culture so that a single tube could be examined and discarded each day to avoid liability of contaminations. Tests were made with normal fresh skimmed milk and with desiccated skimmed milk. The desiccated skimmed milk gave equally good results and was simpler to handle. During a period of 12 weeks, 3 different series of tests were carried out. Since there were no demonstrable changes after the 7th day, the first to the 7th day (Sunday excluded), 14th, and 21st days only are reported. Table 3 gives the combined results.

If we compare the results in table 1 with those in table 3, we will observe that coagulation began with the same P_H as that of lactic acid coagulation, but instead of partial coagulation as given by the lactic acid we have a complete coagulation given by the activity of the organisms in milk. We were therefore compelled to recognize some other agency causing milk coagulation besides that of

acid production by staphylococci. Naturally we turned our attention to enzymes and endeavored to find out under what conditions staphylococci produce enzymes capable of milk coagulation.

TABLE 3
PH OF MILK BEFORE INOCULATION 6.2 TO, BROM-CRESOL-PURPLE

Number and Type of Culture	PH on Number of Days Indicated								
	1	2	3	4	5	Sunday 6	7	14	21
50 St. albus.....	6.1	6.0	6.1	6.2	6.2	..	6.1	6.1	6.1
120 St. albus.....	6.1	6.1	6.1	6.1	6.1	..	6.1	6.1	6.1
122 St. albus.....	6.0	6.0	5.9	5.9	5.9	..	5.9	5.9	5.9
106 St. aureus.....	5.9	5.8	5.8*	5.7*	5.7*	..	5.7*	5.7*	5.7*
14 St. albus.....	5.9*	5.8*	5.7*	5.6*	5.5*	..	5.4*	5.4*	5.4*
109 St. albus.....	5.7*	5.7*	5.7*	5.6*	5.6*	..	5.5*	5.7*	5.7*
61 St. albus.....	6.0*	5.8*	5.8*	5.7*	5.7*	..	5.6*	5.6*	5.6*
118 St. albus.....	6.1	6.1	6.1	6.2	6.4	..	6.4	6.4	6.3

* Curd formation complete for the whole tube.

5. *Determination of Enzymatic Activity.*—Three rapidly coagulating strains were chosen for this work. Three cultures of each were grown in bouillon and also in milk for 96 hours. One bouillon culture was killed by formalin. The remaining bouillon cultures were centrifugalized and both supernatant fluid and sediment were saved and 1% formalin added. Sterility tests were made after 3 days to insure the death of all organisms. The sediment was resuspended in normal salt solution equal to the amount of bouillon poured off. The milk cultures that were clotted were likewise centrifugalized, the whey preserved, the centrifugalized curd resuspended in bouillon, and incubated for 3 days longer. These culture were then centrifugalized and the supernatant bouillon from the milk curd was saved. Both the whey and the milk curd bouillon were sterilized by 1% formalin, as in the preceding bouillon cultures. Sterility tests were made on agar slants the same as for the bouillon cultures. When the sterility of the above broth and milk solutions and suspensions was established, 1 c c, 2 c c, and 3 c c each were added to tubes of milk containing 10 c c and incubated at 37.5 C for 6 days. Daily observations were made. As will be seen, this gave 3 tests for each solution or suspension. Table 4 shows the type of solution and its enzymatic activity on milk.

Table 4 shows that milk was clotted by the sterilized supernatant whey from cultures 14 and 34, and by supernatant bouillon from bouillon-milk curd culture from culture 14. These clots were sub-

cultured on agar slants and incubated for 48 hours but gave no growth. It thus seems reasonable to conclude that the clotting of milk in these cases must be due to the enzyme produced by the staphylococci. No attempt was made to establish any relationship between a proteolytic and a rennet-like enzyme. Other experiments in progress show cultures 14 and 34 to be rapid liquefiers of gelatin and 106 to be a slow liquefier. The results further demonstrate the possibility of the production of an enzyme by staphylococci.

TABLE 4
ENZYMATIC ACTIVITY OF VARIOUS SOLUTIONS ON MILK

	Quantity of Culture 14			Quantity of Culture 34			Quantity of Culture 106		
	1 cc	2 cc	3 cc	1 cc	2 cc	3 cc	1 cc	2 cc	3 cc
Killed broth culture.....	—	—	—	—	—	—	—	—	—
Supernatant centrifugalized broth.....	—	—	—	—	—	—	—	—	—
Saline suspension of sediment from broth culture	—	—	—	—	—	—	—	—	—
Supernatant whey from milk culture....	—	+	+	—	+	—	—	—	—
Supernatant broth from milk curd culture	—	—	+	—	—	—	—	—	—

—, no clot formed; +, solid clot formed.

DISCUSSION AND CONCLUSIONS

The results of this investigation demonstrate that milk is a valuable factor in the classification of staphylococci on account of the various reactions obtained. The phenomenon that bears the most interest in the problem is the question of metabolic activity, particularly among the cultures that caused milk coagulation. Is coagulation due to acid production, direct action of the organisms on the milk, to the production of an enzyme or to some combination of three factors? Judging from the P_H titer of the curdled tube of milk, one might consider it to be a group of factors, such as acid plus direct action, acid plus enzyme action or enzyme action alone. Evidence thus far at hand shows that when acid and direct action of the organisms are eliminated, the specific means are used, as for example, treating the milk with whey from killed coagulated milk cultures, a curd may be produced in sterile milk, thus proving the production of a specific milk curdling enzyme. While further work is in progress on this problem, we feel justified in making the claim that certain strains of staphylococci not related by pigment production are capable of producing a milk curdling enzyme.

THE INFLUENCE OF CHAULMOOGRA OIL ON THE TUBERCLE BACILLUS

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Chaulmoogra oil has been advocated as a remedy for leprosy for many years, and interest in the subject has been greatly renewed by the investigations of Heiser,¹ who has reported a series of cases either apparently cured or greatly improved by intramuscular injections of mixtures of chaulmoogra oil, camphorated oil and resorcin. Subsequent reports by Hopkins,² McCoy and Hollmann,³ Coghill,⁴ Hall,⁵ Cadbury⁶ and Hollmann and Dean,⁷ have indicated that these chaulmoogra mixtures have a definite curative effect in leprosy.

As the *B. leprae* is an acid-fast micro-organism, these results naturally suggest that chaulmoogra oil may be destructive for other acid-fast bacilli, notably the tubercle bacillus, and at the suggestion of Dr. Jay F. Schamberg our experiments were undertaken with a virulent strain of bovine tubercle bacilli. While the work was under way, the excellent paper of Walker and Sweeny⁸ was published. These investigators have found that the sodium salts of the total fatty acids of chaulmoogra oil (chaulmoogrates) possess an extremely high bactericidal and antiseptic activity for the tubercle bacillus in vitro. By incorporating these salts in fluid culture mediums, they have found that dilutions as high as 1:100,000 are bactericidal and 1:1,000,000 may be antiseptic. Furthermore, these chaulmoogrates were found highly specific for acid-fast bacilli, similar experiments with other micro-organisms including *B. coli* and staphylococci, showing an absence of antiseptic activity in dilutions as low as 1:1,000. The authors also give a complete review of the literature and a description of the chemistry of chaulmoogra oil.

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¹ U. S. Pub. Health Rept., 1913, 28, p. 1855; 1914, 29, p. 21; Am. Jour. Trop. Dis. and Prevent. Med., 1914, 2, p. 300.

² New Orleans Med. and Surg. Jour., 1916, 69, p. 223.

³ U. S. Pub. Health Bull., 1916, p. 3.

⁴ Ann. Trop. Med. and Parasit., 1917, 11, p. 205.

⁵ Trop. Dis. Bull., 1919, 13, p. 13.

⁶ China Med. Jour., 1918, 32, p. 226.

⁷ Jour. Cutan. Dis., 1919, 37, p. 367.

⁸ Jour. Infect. Dis., 1920, 26, p. 238.

In 1915, Hernandez⁹ reported that the addition of 2% chaulmoogra oil to culture mediums inhibited the growth of tubercle bacilli and had a favorable influence on tuberculosis in a few patients. Rogers¹⁰ has also suggested the use of sodium chaulmoograte in the treatment of tuberculosis, but, believing that intravenous injections may produce exacerbations, has used instead the sodium salts of the fatty acids of cod-liver oil, believing that the favorable influence of the salts of fatty acids on acid-fast bacteria is nonspecific.

EXPERIMENTS

Our experiments were made with oil expressed cold from seeds of *Taraktogenous kurzii* King* and a strain (H) of bovine tubercle bacilli virulent for guinea-pigs. Since the oil was immiscible with mediums, a special technic was required for determining its possible germicidal activity for the tubercle bacillus. For this purpose the technic worked out by McMaster¹¹ was adopted, the only modification being the substitution of agar slants by Petroff's solid medium and the use of 24-day cultures. Dilutions of chaulmoogra oil were made with sterile paraffin oil, the latter being entirely inert.

In the experiments the tubes of Petroff medium were well drained of water of condensation and inoculated with *B. tuberculosis* over an area of 1 cm. and well above the middle of the tube. These were incubated for 24 days. The oils were then poured over these so as to entirely cover the slant and the tubes were allowed to stand 24 hours in the incubator at 37.5 C. The oils were then poured off and the tubes twice washed out carefully with sterile salt solution. The growths were then transplanted to fresh slants of Petroff medium and incubated for 3 weeks.

With this technic our experiments were entirely negative; even pure undiluted chaulmoogra oil failed to kill all of the tubercle bacilli, as shown in table 1, giving the results of one of these experiments.

These results are in striking contrast to those of Walker and Sweeney,⁸ but our work was conducted with the whole oil while they employed the soluble sodium salts of the total fatty acids. Furthermore, McMaster's technic cannot be regarded as satisfactory for tests of this kind employing the tubercle bacillus because we were obliged

* Prof. Alsberg, Department of Agriculture, Washington, D. C., kindly supplied us with this oil.

⁹ Abstr. in Jour. Am. Med. Assn., 1918, 71, p. 1177.

¹⁰ Brit. Med. Jour., 1919, 1, p. 147.

¹¹ Jour. Infect. Dis., 1919, 24, p. 378.

to use cultures several weeks old which were frequently quite abundant. Even though some of the bacilli, and especially those in immediate contact with the oil, were destroyed, the deeper bacilli may escape and thereby mask any evidence of partial germicidal activity.

The next experiments were made with what may be called a combined in vivo-vitro technic, aiming to bring the oil into more intimate contact with the bacilli and testing for germicidal activity by injecting the mixtures into guinea-pigs.

TABLE 1

THE INFLUENCE OF CHAULMOOGRA OIL ON THE TUBERCLE BACILLUS EMPLOYING THE
McMASTER TEST TUBE METHOD

Dilution of Oil	Results	Dilution of Oil	Results
Undiluted	+	1:3000	—
1:5	+	1:4000	+
1:10	+	1:8000	+
1:100	+	-1:10000	+
1:100	+	-1:10000	+
1:500	—	1:16000	+
1:1000	+	1:24000	+
1:1000	+	1:32000	+
1:1500	+	Paraffin oil (control)	+
1:2000	+	Paraffin oil (control)	+

+ = subcultures showed growth of tubercle bacilli; — = subcultures sterile.

An emulsion of the bacilli was prepared by grinding cultures with sterile paraffin oil and placing 0.1 c.c. of the emulsion in flasks with 10 c.c. of varying dilutions of chaulmoogra oil (diluted with sterile paraffin oil). These mixtures were shaken mechanically with glass beads at room temperature for 18 hours and injected intramuscularly into guinea-pigs in amounts of 0.1 c.c. per 100 gm. of body weight. Controls employing pure paraffin oil alone were included in each experiment. The results were negative; even when the pure undiluted oil was employed, a sufficient number of bacilli escaped destruction to produce tuberculosis in guinea-pigs, as shown in the results of an experiment summarized in table 2.

This technic was rather favorable to any germicidal activity of the oil as it was brought into close contact with the bacilli for 18 hours at room temperature. The number of bacilli was not large and the dose injected into the pigs required 5 to 6 weeks to produce well marked inguinal adenitis and infection of the spleen and abdominal glands. However, even if but few bacilli escaped destruction, the results of inoculation into the young pigs would probably produce tuberculosis; therefore these experiments have shown the absence of

complete gernicidal activity but have yielded no information on the question of partial destruction of the bacilli.

The next experiments were made by infecting young pigs with tubercle bacilli and administering the oil by intramuscular injection.

TABLE 2
THE INFLUENCE OF CHAULMOOGRA OIL ON THE TUBERCLE BACILLUS (IN VITRO-VIVO TECHNIC)

Dilution of Oil Acting on Bacilli	Duration of Lives of Pigs	Necropsies
Undiluted.....	46 days	Generalized tuberculosis
1:5.....	16 days	Tuberculous adenitis
1:5.....	46 days	Generalized tuberculosis
1:10.....	37 days	Generalized tuberculosis
1:10.....	44 days	Generalized tuberculosis
1:100.....	49 days	Generalized tuberculosis
1:100.....	55 days	Generalized tuberculosis
1:1000.....	16 days	Tuberculous adenitis
1:1000.....	16 days	Tuberculous adenitis
1:10,000.....	55 days	Generalized tuberculosis
1:10,000.....	34 days	Generalized tuberculosis
1:100,000.....	20 days	Generalized tuberculosis
1:1,000,000.....	16 days	Tuberculous adenitis
No oil; culture control.....	46 days	Generalized tuberculosis
No bacilli; oil control.....	Lived indefinitely	No changes

TABLE 3
THE INFLUENCE OF CHAULMOOGRA OIL ON GUINEA-PIGS INFECTED WITH TUBERCULOSIS

Administration of Oil	Duration of Life	Necropsies
First dose before infection; three doses later...	26 days	Tuberculous adenitis
First dose before infection; four doses later...	32 days	Tuberculous adenitis
First dose at time of infection; two doses later	26 days	Tuberculous adenitis
First dose at time of infection; nine doses later	57 days	Tuberculous adenitis
First dose one day after infection; three doses later	29 days	Generalized tubereulosis
First dose one week after infection; seven doses later	70 days	Generalized tuberculosis
Not infected; eleven doses of oil (control).....	Alive	Chronic myositis
Not infected; nine doses of oil (control).....	Alive	Chronic myositis
Infected (control); no oil.....	76 days	Generalized tuberculosis
Infected (control); no oil.....	22 days	Generalized tuberculosis

The inguinal glands of tuberculous pigs (5 weeks' infection) were emulsified with sufficient sterile salt solution to show only a few bacilli in stained smears of a loopful. These emulsions were then paper filtered and 0.2 c c injected subcutaneously into the abdomen. The untreated controls infected in this manner lived from 22 to 76 days before succumbing with general miliary tuberculosis.

The chaulmoogra oil was administered by intramuscular injection at weekly intervals in doses of 0.2 c c per 100 gm. of body weight.

Some pigs received the oil prior to infection, others received the first dose simultaneously with infection and others at varying intervals after infection, as shown in results of an experiment summarized in table 3.

The results have been of a negative character; when the oil was administered before or simultaneously with infection, the degree or extent of infection appeared to be confined to the neighboring glands in contrast to the generalized miliary tuberculosis which usually developed when the infection was given a longer start before the administration of oil was begun. As a general rule, multiple injections of the oil produced localized inflammatory changes at the sites of injection.

SUMMARY

Undiluted chaulmoogra oil (*Taraktogenos kurzii* King) and dilutions in paraffin oil, had no appreciable germicidal influence in vitro on a strain of bovine tubercle bacilli, according to the results observed with the technic employed in these experiments. The sodium salts of the total acids of this oil (chaulmoogrates) were not included in this study.

Undiluted and diluted chaulmoogra oil had no appreciable germicidal effect on virulent tubercle bacilli as determined by an in vitro-vivo method employing guinea-pigs.

Chaulmoogra oil in doses of 0.2 c c per 100 gm. of body weight administered by intramuscular injection at weekly intervals (equivalent to 2 c c per kilo or 120 c c per 60 kilos) had none or but slight effect on the course of tuberculosis in infected guinea-pigs.

Chaulmoogra oil is relatively nontoxic for guinea-pigs; animals have borne at least 11 intramuscular injections of 0.2 c c per 100 gm. without deleterious effect except localized inflammatory changes at the sites of injection.

THE CHARACTERISTICS OF THE MICROCOLONIES OF SOME PATHOGENIC COCCI*

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A new method for the study of bacteria was recently described by one of us,¹ in which it was pointed out that it was possible to dry down and stain colonies of bacteria only a few hours old. In these microcolonies the cell form and structure are quite as well brought out as they would be in a cover glass preparation and in addition the relation of cell to cell is easily studied. These colonies appear to show distinctive characters and the method promises to be of material aid in the study and recognition of bacteria.

It is proposed in this paper to describe the results of a study of the microcolonies of some staphylococci, streptococci and pneumococci.

METHOD

The culture medium for the staphylococci was sterile milk and 1% nutrient agar (dehydrated Difco) equal parts. For the streptococci and pneumococci, 0.5 c c horse or rabbit serum or whole blood was added to a tube of the milk and agar mixture (6 to 12%). The organisms were all given a preliminary culture in a liquid medium for a few hours.

The "little plates" were made by placing two small drops of the liquefied medium on a glass slide which had just been sterilized in the flame. Into one drop a loopful of the preliminary culture was put and spread about into a thin film. The loop without resterilization was then used to spread the second drop. In this way two "little plates" were formed, one of which was a dilution of the other. They were then incubated in a moist chamber for 4 hours. The form of moist chamber is immaterial. For a few slides, Petri dishes may be used. In this case a piece of wet filter paper is placed in the bottom and the slides rested on glass rods or match sticks. A convenient form is a "moist chamber cabinet" designed especially for the purpose (Central Scientific Company of Chicago). An incubation period of from 4 to 6

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¹ Frost, Jour. Am. Med. Assn., 1919, 72, p. 323.

hours seems best. Younger colonies are likely not to be characteristic—older ones are frequently too dense to reveal the structure. When removed from the moist chamber they were dried down very rapidly on a steam plate and then put in a stain made thus: thionine blue, 1 gm.; phenol, 2.5 gm.; distilled water, 400 gm.; and glacial acetic acid, 20 c c.

Two minutes was sufficient, but a longer time does not cause over-staining. The slides were thoroughly washed in tap water and dried either at room temperature or on the steam plate. They were examined under the immersion objective by putting the oil directly on the preparation.

The photomicrographs of the colonies were made by means of a horizontal camera suspended on springs. The source of light was 1,000 watts mazda with condensed filament. The Wratten and Wainwright "M" Plates together with the Wratten filter B and E were used.

The following cultures were studied:

(a) *Staphylococcus pyogenes* var. *aureus*, one strain.

Staphylococcus pyogenes var. *albus*, one strain.

(b) Hemolytic *Streptococci*

Streptococcus pyogenes, one strain.

Streptococcus epidemicus, one strain.

Streptococcus alactosus, one strain.

Streptococcus hemolyticus, none.

(c) Nonhemolytic *Streptococci*

Strain *mitis*, 11 strains.

Strain *fecalis*, 3 strains.

Strain *saliarius*, 2 strains.

Strain unidentified, 9 strains.

(d) *Pneumococci*

Type 1, 9 strains.

Type 2, 9 strains.

Type 3, 10 strains.

Type 4, 3 strains.

MICROCOLONY CHARACTERISTICS OF THE *STAPHYLOCOCCUS* GENUS

The microcolonies of the *staphylococcus* genus are of medium size, with a more or less dense, compact center. At the edge of the colony are seen flattened or biscuit-shaped diplococci, frequently in a tetrad

grouping. The mode of division of the cell seems to be in two planes, i. e., at right angles to each other.

The albus colony seems to be more open and shows the typical tetrad grouping more plainly than the aureus, which, on the other hand, has a dense center with a few biscuit-shaped diplococci, often in tetrads, on the border of the colony.

MICROCOLONY CHARACTERISTICS OF THE STREPTOCOCCUS GENUS

The streptococcus genus is usually divided into two groups, the hemolytic and the nonhemolytic. Ruediger² and afterward Holman³ have noted some morphologic differences. The hemolytic group, he says, is characterized by the formation of closely packed, frequently disk-shaped cocci in chains which have the appearance of having been compressed at right angles to the axis of the chains. On the other hand, it was noted that the nonhemolytic group is characterized by the appearance of elongated clearly marked pairs, giving the chains the appearance of having been stretched.

In agreement with Ruediger and Holman, these general differences have been observed in the study of a number of strains and are obvious in the photomicrographs.

The microcolonies of the hemolytic group show the single cocci in chains. The individuals are frequently not spherical, but rather compressed at right angles to the axis of the chains. The closely packed, frequently disk-shaped, individual cocci give them a tightly bound, firm appearance. Some microcolonies of the hemolytic group show a somewhat dense center with some disk-shaped or spherical diplococci at the outer edge of the colony. The colonies of this type have no chains, but the cocci at the border of these colonies are quite noticeably spherical and occur in pairs. The microcolonies are large, some are circular in form—especially the compact colony—while others are angular in form and seem to spread in various directions with the interlacing of the chains.

The microcolonies of the nonhemolytic group are characterized by the elongated diplococci which are distinctly separated from the next pair, but are joined together in a loose chain. The separation of the

² Jour. Infect. Dis., 1906, 3, p. 761.

³ Jour. Med. Research, 1915, 34, p. 392.

diplococci gives the chains the appearance of having been stretched. Some microcolonies of this group show a rather dense center with a few elongated diplococci at the edge of the colony, with no tendency to form chains.

The microcolonies of this group are also large; some are angular with the interlacing of chains, while others are somewhat circular with the dense center and no chains. Colonies of this type are difficult to distinguish from the microcolonies of the dense type of the hemolytic group.

MICROCOLONY CHARACTERISTICS OF THE PNEUMOCOCCUS GENUS

The microcolonies of the pneumococcus genus are small and open with the cells arranged in a free and distributed manner. The lancet-shaped cells are grouped together as diplococci. There is a slight tendency toward chain formation in type 3, which by some is called *Streptococcus mucosus* and in type 4 which includes various unknown pneumococci.

Contrasting the pneumococcus microcolonies with those of the streptococci: In general the pneumococcus colonies are very small, composed of lancet-shaped diplococci, which may occur in short chains or alone. The small size of the colony is one of the noticeable characteristics. The streptococcus microcolonies are considerably larger, with the diplococci arranged in closely packed chains in some strains or in loosely connected chains in others.

DOES ANIMAL PASSAGE INFLUENCE THE CHARACTER OF THE COLONY?

In the study of the pneumococcus, the question was raised: Is the colony formation altered by animal passage or, in other words, would the colonies be different if the organisms came directly from a diseased condition? In order to study this, white mice were inoculated intraperitoneally and the organism recovered from the heart blood. Cultures of several strains on blood agar were used, and "little plates" of the organism from the heart blood were grown and their morphologic characteristics determined. The character of the microcolony does not seem to be altered by animal passage, except that in a few cases the colonies are somewhat smaller than the microcolonies of the same organism before animal passage.

EXPLANATION OF PLATE I

Colonies of Staphylococci 5 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 1.—Colony of Staph. pyogenes, var. albus.

Fig. 2.—Colony of Staph. pyogenes, var. aureus.

Colonies of Nonhemolytic Streptococci 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 3.—Colony of Strep. mitis.

Fig. 4.—Colony of Strep. mitis.

Fig. 5.—Colony of Strep. fecalis.

Fig. 6.—Colony of Strep. salvarius.

Fig. 7.—Colony of strep. from case of ozena.

Fig. 8.—Colony of strep. from case of scarlatina.

Fig. 9.—Colony of strep. from case of acute articular rheumatism.

Colonies of Hemolytic Streptococci 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 10.—Colony of Strep. pyogenes.

Fig. 11.—Colony of Strep. epidemicus.

Fig. 12.—Colony of Strep. alactosus.

Fig. 13.—Colony of a hemolytic streptococcus.

Fig. 14.—Same organism as in fig. 13, but a deep colony.

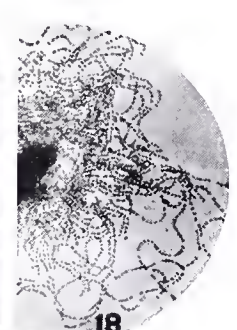
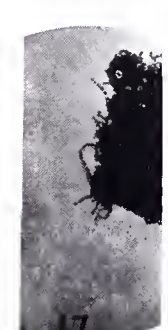
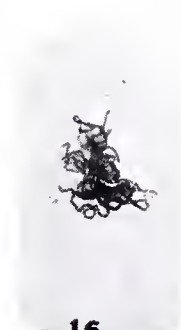
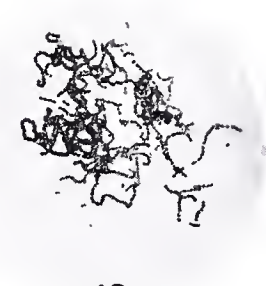
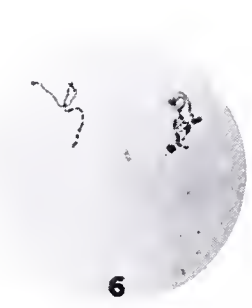
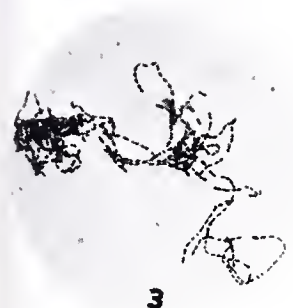
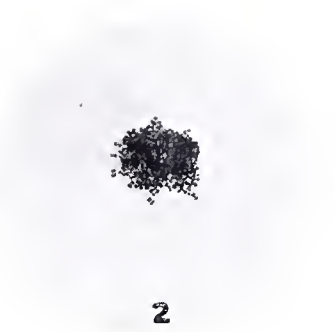
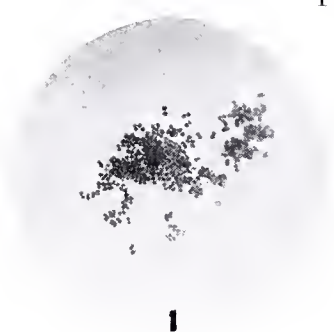
Fig. 15.—Colony of a hemolytic streptococcus.

Fig. 16.—Colony of a hemolytic streptococcus from case of otitis.

Fig. 17.—Deep colony of a hemolytic streptococcus from case of mastitis.

Fig. 18.—Same organism as in fig. 17 but a surface colony.

PLATE 1



EXPLANATION OF PLATE II

Colonies of Pneumococci Type 1, 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 1.—Colony of pneumococcus type 1.

Fig. 2.—Colony of pneumococcus type 1.

Fig. 3.—Colony of pneumococcus type 1 from heart blood of mouse. Culture same as in fig. 2.

Colonies of pneumococci type 2, 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 4.—Colony of pneumococcus type 2.

Fig. 5.—Colony of pneumococcus type 2 from heart blood of mouse; culture same as in fig. 4.

Fig. 6.—Colony of pneumococcus type 2.

Fig. 7.—Colony of pneumococcus type 2 from heart blood of mouse; culture same as used in fig. 6.

Colonies of pneumococcus type 3, 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 8.—Colony of pneumococcus type 3.

Fig. 9.—Colony of pneumococcus type 3 from heart blood of mouse; same strain as in fig. 8.

Fig. 10.—Colony of pneumococcus type 3.

Fig. 11.—Colony of pneumococcus type 3 from heart blood of mouse; culture same as in fig. 10.

Fig. 12.—Colony of pneumococcus type 3.

Fig. 13.—Colony of pneumococcus type 3 from heart blood of mouse; culture same as in fig. 12.

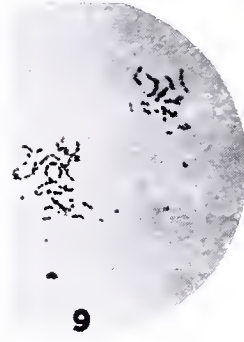
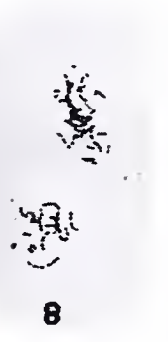
Fig. 14.—Colony of pneumococcus type 3 from heart blood of mouse 2nd passage; culture same as in fig. 12.

Colonies of pneumococcus type 4, 4 Hours Old Incubated at $37\frac{1}{2}$ C.

Fig. 15.—Colony of pneumococcus type 4.

Fig. 16.—Colony of pneumococcus type 4 from heart blood of mouse; culture same as in fig. 15.

PLATE II



CHANGES IN LEUKOCYTES AND ALKALI RESERVE OF BLOOD IN EXPERIMENTAL INFECTIONS

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Methods for estimating quantitatively the alkali reserve of the blood depend on determining the carbon dioxid binding power of either whole blood or blood plasma after saturation with air or gas mixtures containing a definite concentration of carbon dioxid. The results, while expressing only a part of the entire alkali reserve, are accepted generally as an index of the chemical condition of the blood, a diminution below the normal carbon dioxid binding power being regarded as acidosis. Diminished alkali reserve of the blood has been observed in the acidosis of diabetes and uremia, also in pneumonia and in other acute infectious diseases. There seems to be no observations at hand in regard to the alkali reserve changes in the blood in experimental infections with parallel observations of the variation in the number of leukocytes. The results of such a study in rabbits are contained in this report.

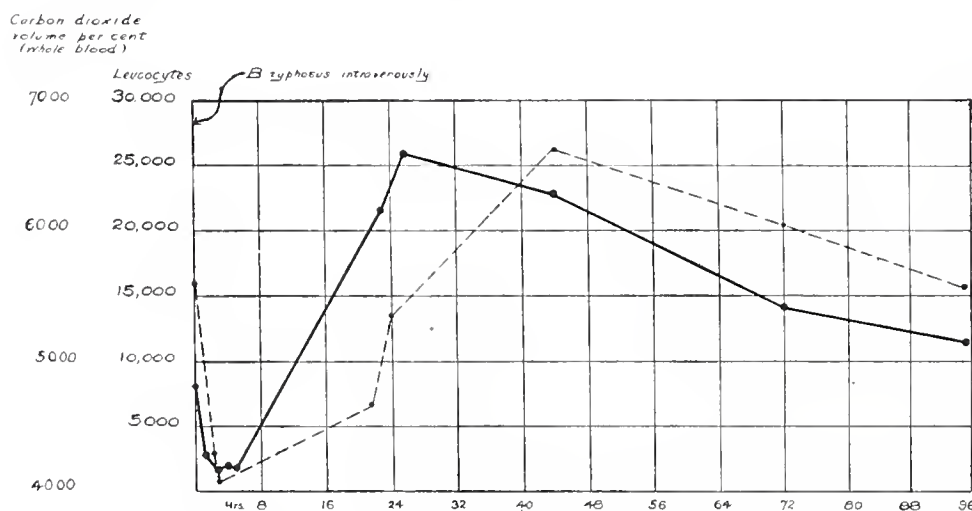
Living cultures of various bacteria were injected intravenously into rabbits, and at short intervals thereafter the animals were bled from the ear vein, and the alkali reserve of the whole blood was determined with a portion of the blood, the number of leukocytes with another portion taken directly from the bleeding vessel. Injections with the following bacteria were made: *B. typhosus*, *B. dysenteriae* (Flexner), *B. coli*, *Streptococcus hemolyticus*, *Streptococcus viridans*, *B. diphtheriae*, *B. pneumoniae*, and *B. welchii*. These bacteria were grown in pure culture on suitable plain or blood-agar slants, the growth after 18 to 24 hours' incubation suspended in sterile normal salt solution, and the suspension injected intravenously, the fluid volume injected not exceeding 2.5 c.c. The alkali reserve of the whole blood was determined according to the Van Slyke and Cullen¹ method after equilibration with air containing 5.5% carbon dioxid, and the readings obtained were corrected for 0 C. and 760 mm. barometric pressure.

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¹ J. Biol. Chem., 1917, 30, p. 289.

Within 2 to 4 hours after the injection of the suspension of the bacteria, the leukocytes of the blood rapidly diminish in number, in some rabbits decreasing to 1,000 to 2,000 per cmm. Coincidentally, the carbon dioxide binding power of the blood diminishes rapidly, its lowest level being reached shortly after the minimum attained by the leukocytes. During the succeeding 12 to 18 hours there is a rapid increase in the number of leukocytes, and a return to normal of the blood alkali reserve, the latter lagging somewhat behind the former. A marked leukocytosis is preceded by a marked and comparatively long continued depression of the blood alkali reserve. This low alkali reserve is followed by a rebound to a higher than normal value (alkalosis) of short duration, and then a gradual return to normal with the return to normal of the number of leukocytes.



Illustrating the changes in the number of leukocytes and in the alkali reserve of the blood following an intravenous injection of living typhoid bacilli. The continuous black line indicates leukocytes; the broken line the alkali reserve.

The recovery of the alkali reserve and the rise in the number of leukocytes of the blood is not always parallel. The curves usually are parallel when the return to normal of each takes place within 48 to 72 hours. In experiments in which the maximum leukocytosis is not accompanied or followed shortly by a prompt return to normal of the blood alkali reserve, the latter remains depressed and the leukocytes continue high. Later, with the alkali reserve returning to normal, the number of leukocytes decreases and the curves tend to become parallel. Rabbits with only a slight depression of their blood alkali reserve have little or no variation in the number of their leukocytes.

RESULTS OF EXPERIMENTS WITH VARIOUS BACTERIA

Time	Colon Bacillus		Dysentery Bacillus		Typhoid Bacillus		Hemolytic Streptococcus		Streptococcus Viridans		Pneumo-coccus		Staphylo-coccus		Diphtheria Bacillus		Friedländer Bacillus		Bacillus Welchii	
	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes	Alkali Reserve	Leuko-cytes
Before infection	64.88	11,200	65.58	7,500	67.46	12,100	68.76	9,800	55.78	9,500	55.72	7,700	63.97	7,200	66.04	11,300	58.94	8,400	66.81	15,000
1 hour	64.24	6,000	53.97	3,400	5,500	64.37	6,300	48.96	2,500
2 hours	42.83	2,000	50.65	1,900	39.30	3,600	55.29	6,400	50.26	61.21	61.20	51.64	1,000	66.81	10,800
3 hours	41.18	2,700	19.41	9,300	46.64	41.29	1,400	53.97	44.35	800
4 hours	45.74	3,700	22.75	7,700	50.26	3,900	56.64	10,100	45.21	1,000
5 hours	50.38	12,000	43.13	3,100	32.09	7,000	48.99	5,000	49.29	4,100	45.69	2,100
6 hours	46.70	8,400	37.57	3,500	25.75	14,500	25.75	48.49	2,500
7 hours	50.48	8,000	42.26	2,300	dead	48.49	7,000	62.17	7,200
8 hours	46.01	9,300	43.95	10,400
9 hours
10 hours
19 hours	48.55	27,700	55.21	12,900
20 hours
21 hours	50.39	25,200	80.50	20,000	55.11	28,700	57.17	8,300	65.85	13,000
22 hours	48.55	23,000	51.33	20,300	57.47	24,800
23 hours	54.43	10,500
24 hours	67.59	20,000
26 hours	55.34	19,000	63.02	12,400	58.63	10,300	59.31	9,000	63.17	17,000
2 days	67.44	24,500	61.79	10,500
3 days	65.47	21,100	67.30	12,800
4 days	45.79	28,000	63.60	14,000	54.82	10,200	52.02	23,400
5 days	47.04	22,000	50.89	10,200	65.07	10,500
6 days
7 days	55.00	18,000	59.83	11,600
8 days	56.90	14,000
9 days	65.34	24,300
12 days	57.72	15,000
12 days	57.72	9,000

The chart represents graphically the alkali reserve changes and the variation in the number of leukocytes of the blood following an injection of typhoid bacteria. The curves illustrating these changes in rabbits injected with other bacteria are comparable. The table contains the data obtained in the individual experiments with various bacteria other than the one given in the chart.

Control rabbits receiving sterile salt solution or broth up to 6 c.c. intravenously manifest no change in their blood alkali reserve or in the number of leukocytes. Other factors such as handling of the animals, environment, and the amount of blood drawn have been controlled and found to have no results such as those given for the injections of bacteria.

DISCUSSION

The mobilization of leukocytes at the site of infected tissues because of chemical substances produced or liberated by bacteria is well known. Many substances have been found to exert locally a positive chemotactic influence on leukocytes, others a negative.² The results obtained in my experiments indicate that a general leukocytosis and leukopenia are accompanied by definite chemical changes of the blood. It is possible that the factors lowering the alkali reserve, or that the lowered alkali reserve itself (acidosis) furnish the chemical stimulus necessary for the production of leukocytosis. The results obtained seem to indicate that the degree of leukocytosis is in ratio not so much with the extent of the initial leukopenia as it is with the degree of alkali reserve depression and the length of time during which it is depressed.

CONCLUSIONS

The intravenous injection of living bacteria into rabbits usually causes, within a period of 2 to 4 hours, a leukopenia and a diminution of the blood alkali reserve. There follows a rise in the number of leukocytes and a return to normal of the alkali reserve. There may be a short period of alkalosis. The minimal and maximal alkali reserve levels are reached after a corresponding leukopenia and leukocytosis. Continued low alkali reserve is accompanied by a persistently high leukocytosis. With the rise to normal of the alkali reserve there is a decline in the number of leukocytes. It is suggested that diminution of the alkali reserve, or the factors associated with this depression, may afford the chemical stimulus necessary for the subsequent leukocytosis.

² Wells, H. G.: *Chemical Pathology*, 1920, p. 250.

THE EFFECT OF MUSTARD GAS (DICHLORETHYL-SULPHID) ON ANTIBODY FORMATION

LUDVIG HEKTOEN AND H. J. CORPER

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Mustard gas was discovered by Victor Meyer¹ but its poisonous effects attracted little attention until 1917 when it was used by the Germans at Ypres, and since then much has been written about it from various points of view.²

Meyer noted that this substance had a specific toxic action on the skin, conjunctiva and respiratory tract, and he concluded that the most severe action develops only after its entrance into the blood. The toxicity, he found, depends on the chlorine content, the monochlorethylsulphid being less toxic than the dichlorethylsulphid. Man seems to be more susceptible than animals and to reveal a greater individual difference, so far as the skin is concerned.³ The extreme toxicity of mustard gas is shown by the fact that the human eye may react after an exposure of less than one hour to one part in 10,000,000.⁴ That the hematopoietic system is affected is indicated by the leukopenia quickly produced by the gas. Krumbhaar and Krumbhaar,⁵ from observations on 108 victims whose blood was studied and in 75 necropsies, in 55 of which the bone marrow was examined, conclude:

"1. Yellow cross or mustard gas exerts on the bone marrow a direct toxic action, which, by depleting the leukocytes of the circulation, has an important bearing on the inability to resist secondary infection that is found in that form of gas poisoning. 2. This toxic action on the bone marrow is shown not only by small areas of necrosis, but by an inhibition of the regenerative process (chiefly of the leukogenetic series). 3. Not only is the amount of regenerative hyperplasia inadequate to the severity of the process (as compared with the marrow hyperplasia of various acute infections), but also the quality is inferior, that is, the great majority of the homopoietic cells present are of immature types."

They found also that the period of highest mortality in man coincides with the most severe leukopenia, and regard the action on the bone marrow as similar to that of benzene.

More recently Pappenheimer and Vance,⁶ in a study of the effects of intravenous injection of dichlorethylsulphid in rabbits, found the lethal dose thus given to be 0.005 to 0.01 gm. per kilo. Animals thus treated and dying within

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¹ Ber. d. deutsch. chem. Gesellsch., 1886, 19, p. 3259, and 1887, 20, p. 1729.

² For a complete bibliography to 1919 the excellent monograph "Mustard Gas Poisoning," by A. S. Warthin and C. V. Weller, 1919, may be consulted.

³ Eyster, J. A. E., and Maver, Mary E.: Jour. Pharm. and Exper. Therap., 1920, 15, p. 95.

⁴ Reed, C. I.: Ibid., 1920, 15, p. 77.

⁵ Jour. Med. Res., 1919, 40, p. 497.

⁶ J. Exper. Med., 1920, 31, p. 72.

24 hours showed extensive hemorrhages and edema of the lungs, and about one-third presented severe lesions of the intestinal tract. Dichlorethylsulphid injected intravenously was specifically poisonous for the hematopoietic tissues producing severe lesions in the marrow with a marked diminution of circulating leukocytes. The granular cells of the marrow seemed to be more sensitive than the lymphoid cells and erythrocytes. They found that this effect of mustard gas on the blood and hematopoietic tissues was not due to the admixture of nitrobenzene or chlorobenzene. They used a freshly prepared suspension in 30% alcohol in distilled water made from a 10% solution in absolute alcohol, and to avoid hydrolysis of the mustard gas, which occurs rapidly in aqueous suspensions, the injection was made immediately after dilution. Warthin and Weller confirm these observations.

Lynch, Smith and Marshall⁷ found a dose of 6 mg. per kilo injected intravenously in aqueous solution to be fatal for dogs. Their aqueous solutions proved markedly unstable, 15% hydrolyzing in 10 minutes at 10 C., while at 37.5 C., over 97% was decomposed in the same time. They found that mustard gas is excreted in the urine, in part at least, as dihydroxyethylsulphid, a comparatively nontoxic body, and advance the theory of action that the dichlorethylsulphid penetrates the cells, and in the aqueous phase of the cell hydrolyzes to hydrochloric acid, which is responsible for the damage.

In the early studies on the circulating leukocytes after mustard gas poisoning, the leukopenia was apparently not noted, but Krumbhaar⁸ called attention to this in man, especially to be noted in severe or fatal cases. At first there is an increase in both the red and white count (in individual cases as high as 36,000 and averaging in his series about 12,000) not to be attributed to the increased blood concentration. The Arneth scale is distinctly shifted to the left and then shifts to the right indicating an exhaustion of the leukocyte forming centers. If death does not interrupt the downward course, an extreme degree of leukopenia (at the expense of the polymorphonuclears) may be reached. With recovery, the leukocytes gradually rise to normal or slightly above. An initial rise in erythrocytes is later replaced by a moderate anemia. In the later leukopenic stage, the blood platelets become sparser. Pappenheimer and Vance⁶ found unexplained variations in the percentage leukocyte counts of gassed rabbits. In rabbits surviving more than 24 hours after a single injection of 0.005 to 0.01 gm. of mustard gas per kilo there was a pronounced fall in the number of circulating leukocytes. Even in the extreme leukopenia, the rare leukocytes were normal morphologically. There was an absolute and percentage increase in the polymorphonuclears, which fell rapidly with the onset of the leukopenia. In the terminal stages the polymorphonuclears disappeared from the peripheral blood. Sometimes the initial fall in number and percentage of polymorphonuclears is followed by a relative and percentage increase. This secondary rise is associated with the appearance of "unripe" forms in considerable numbers. The leukopenia is accompanied by a relative lymphocytosis. The absolute number of lymphocytes is diminished in the later stages and lags behind that of the granular cells in the cases in which regeneration occurs. There is a percentage increase in the large mononuclear cells, but their absolute number is unchanged or diminished. There is a moderate anisocytosis of the erythrocytes in the terminal stages and during recovery numerous normoblasts appear.

⁷ Jour. Pharm. and Exper. Therap., 1918-19, 12, p. 265.

⁸ Jour. Am. Med. Assn., 1919, 72, p. 39.

So far as we are aware, no observations have been made on the effects of mustard gas on antibody formation. In view of its action on the marrow, which resembles to some extent the actions of benzene, of the roentgen ray and of thorium X, all of which have been found to interfere with the production of antibodies, it seemed to us that it might be of interest to study mustard gas from this point of view also. Accordingly, the following experiments have been made on rabbits and dogs, the technic for estimating the antibody content of the serum being the same as in the previous experiments along the same general line.⁹ In the tables the figures give the highest active dilutions of serum with respect to lysin and agglutinin and the highest dilution of antigenic serum giving the precipitate reaction with the serum of animals experimented with.

The mustard gas was obtained from Dr. J. A. E. Eyster of the University of Wisconsin. On account of the rapid hydrolysis in watery solution, we used 0.1% solutions in 50% glycerol, which remain stable at least for some hours. Preliminary tests showed that 0.005 c c of mustard gas in 50% of glycerol per kilo is lethal for rabbits when injected intravenously and that 0.003 c c per kilo is lethal for dogs.

EXPER. 1.—(Belgian hares weighing from 3½ to 4½ kilograms.) Rabbits were given various doses of mustard gas intravenously a few days before and after, as well as the same time as they received 30 c c of sheep blood intraperitoneally. The titer of the serum in antishoop lysin and precipitin was determined in the usual way, at first daily and later at longer intervals. While this experiment was in progress, an epidemic of snuffles broke out among the rabbits in the laboratory on account of which the results may have been affected to a certain extent, but in view of the results of control observations and of later experiments, the effect of the epidemic evidently was of minor importance at the most. In the animals that received mustard gas (0.005, 0.001, 0.0001 c c) 4 days before the antigen, in all cases less lysin and precipitin were produced than in the controls. The lysin curve in the animals that received mustard gas ran about the usual course, but the latent period of the precipitin curve was much longer than usual; in one case no precipitin appeared until the 13th day and in two other animals precipitin was not demonstrable until the 9th day, while in the controls it was demonstrable on the 6th or 7th day. The effects outlined were obtained in about the same degree regardless of the amount of mustard gas, but the reduction in the number of leukocytes in the blood was greatest by far in the rabbits receiving the largest amount of mustard gas (0.005). Practically the same effects were obtained in the animals that received the mustard gas at the same time as the antigen. Here also a prolongation of the latent period in precipitin production was noticeable. Given 4 days after the antigen, the mustard gas still seemed to have some restraining effect on antibody production. The results are illustrated in table 1.

⁹ Hektoen: *J. Infect. Dis.*, 1916, 19, p. 69; 1915, 17, p. 415; 1918, 22, p. 28. Hektoen and Corper: *ibid.*, 1920, 26, p. 330.

EXPER. 2.—In this experiment, also in experiments on rabbits (domestic), only one dose, namely, 0.001 cc of mustard gas was given and the amount of antigen was 20 cc. No epidemic influenced the results in any way. The injection of 0.001 cc of mustard gas 5 days before the antigen had a more marked effect on the production of lysin and precipitin, the lysin curve being especially irregular, than in the animals that received the mustard gas with the antigen or 5 days later than the antigen (table 2).

TABLE 1
EFFECTS OF MUSTARD GAS ON ANTIBODY FORMATION IN RABBITS

Days After Injection of Sheep Blood (30 cc)	Mustard Gas, 0.005 cc Given 4 Days Before Injection of Sheep Blood		Mustard Gas, 0.005 cc Given at Same Time as Injection of Sheep Blood		Mustard Gas, 0.001 cc Given 4 Days After Injection of Sheep Blood		Control	
	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
1	192	0	0	0	96	0		
2	192	0	96	0	192	0	384	0
3	1,536	0	48	0	192	0		
4	1,536	0	192	0	384	0	1,536	0
5	3,072	0	768	0	1,536	0		
6	3,072	0	768	0	1,536	0		
7	3,072	0	768	0	1,536	400	3,072	3,200
9	1,536	0	768	400	6,144	800	6,144	3,200
11	3,072	0	384	200	6,144	3,200	12,288	3,200
13	3,072	1,600	48	200	6,144	1,600	6,144	6,400
15	3,072	1,600	24	0	6,144	3,200	6,144	6,400
18	3,072	1,600	96	0	6,144	1,600	6,144	6,400
20	3,072	6,400	192	0	6,144	1,600	6,144	6,400
22	3,072	6,400	192	0	6,144	1,600	6,144	6,400
25	384	6,400	192	0	6,144	6,400
27	384	1,600	96	0				

TABLE 2
EFFECT OF 0.001 CC OF MUSTARD GAS ON ANTIBODY FORMATION IN RABBITS

Days After Injection of Sheep Blood	Mustard Gas Given 5 Days Before Injection of Sheep Blood		Mustard Gas Given at Same Time as Injection of Sheep Blood		Mustard Gas Given 5 Days After Injection of Sheep Blood		Control	
	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin	Lysin	Precipitin
3	384	0	768	0	384	0	48	0
5	3,072	0	12,288	0	3,072	0	1,536	0
7	96	0	12,288	50	12,288	800	12,288	400
9	384	0	6,144	800	12,288	800	12,288	1,600
11	768	0	6,144	1,600	12,288	6,400	12,288	6,400
13	12,288	0	12,288	1,600	3,072	6,400	6,144	6,400
15	6,144	800	12,288	3,200	3,072	6,400	6,144	6,400
16	6,144	1,600	6,144	3,200	3,072	1,600	6,144	3,200
19	12,288	1,600	6,144	800	3,072	1,600	3,072	1,600
21	12,288	0	3,072	200	1,036	1,600	3,072	1,600
24	6,144	0	3,072	0	1,536	800	1,536	800

EXPER. 3.—The study was made on dogs and the antigen was sheep blood, 30 cc being injected intraperitoneally. The amount of mustard gas given was 0.5 cc 0.1% solution per kilo of weight. The titer of lysin only was determined, the serum being heated to 56 C. for 30 minutes and complemented in each case with 0.0125 cc of fresh guinea-pig serum, each mixture containing 2 cc of 5% sheep blood (washed), the requisite amount of heated dog serum, complement and salt solution to make 6 cc. The dogs that received mustard

gas 7 and 3 days before the antigen and those receiving it simultaneously with the antigen produced uniformly less lysin than the controls and the dogs that received mustard gas 3 and 7 days after the antigen. It is not possible to say whether in the last case the production of lysin was affected much or not. In all cases the fairly typical curves were described.

TABLE 3
EFFECT OF MUSTARD GAS ON LYSIN PRODUCTION IN DOGS INJECTED INTRAPERITONEALLY
WITH 30 C.C. OF SHEEP BLOOD

Days After Injection of Sheep Blood	Mustard Gas Given 3 Days Before Injection of Sheep Blood	Mustard Gas Given at Same Time as Injection of Sheep Blood	Mustard Gas Given 3 Days After Injection of Sheep Blood	Controls
2	48	48	48	24
4	48	192	384	96
6	192	768	768	384
8	1,536	1,536	6,144	384
10	12,288	6,144	768
12	3,072	12,288	6,144	3,072
14	3,072	3,072	3,072	12,288
16	3,072	1,536	1,536	12,288
18	1,536	1,536	1,536	6,144
20	1,536	1,526	1,536	3,072
22	1,536	1,536	1,536	3,072
24	1,536	1,536	1,536	3,072
26	768	1,536	1,536	1,536
28	768	Died	1,536	768
30	768		1,536	768
32	768		768	384
34	384		768	192
36	384		384	96
38	192		384	192

TABLE 4
EFFECT OF MUSTARD GAS ON AGGLUTININ PRODUCTION IN DOGS INJECTED INTRAVENOUSLY
WITH 1 C.C. OF A 10% SUSPENSION OF RAT BLOOD PER KILO

Days After Injection of Rat Blood	Mustard Gas Given 7 Days Before Injection of Rat Blood	Mustard Gas Given at Same Time as Injection of Rat Blood	Mustard Gas Given 4 Days After Injection of Rat Blood	Controls
1	58	48	96	48
3	192	48	192	192
5	192	384	384	768
7	96	384	384	768
9	192	768	768	1,536
11	94	768	768	1,536
13	48	384	384	1,536
15	96	192	384	768
17	48	192	192	768
19	48	96	192	384
21	48	96	96	384
23	48	192	96	384
27	48	192	96	192
31		192	96	192
36		192	96	192

EXPER. 4.—In this experiment dogs were injected intravenously with 1 c.c. of a 10% suspension of rat blood per kilo of weight and the titer of the resulting agglutinin for rat corpuscles was determined. As before, mustard gas (0.5 c.c. 0.1% per kilo) was given intravenously before and after and also simultaneously with the antigen injection. The results were definite. In the dogs given mustard gas before the antigen there was hardly any new

production of agglutinin; in the dogs injected with mustard gas and rat corpuscles at the same time the agglutinin production was much less than in the controls; and in the dogs injected with mustard gas after the injection of the antigen there was little, if any, interference with agglutinin accumulation in the blood as compared with the controls (table 4).

TABLE 5
LEUKOCYTES IN DOGS GIVEN LETHAL AND NONLETHAL INTRAVENOUS INJECTIONS OF
MUSTARD GAS IN 50% GLYCEROL

Amount of Mustard Gas Given	Dog	Time of Blood Examination in Relation to Mustard Gas Injection	Leukocytes on the Basis of 100 Cells Counted									
			Total per C.Mm.	Polymorpho-nuclears (Arneth Scale)					Eosino-phils	Baso-phils	Mono-nuclears	
				I	II	III	IV	V			Large	Small
0.25 c c 0.2% in 50% glycerol per kilo	1 12 kilos in wt.	Before	6,600	1	22	30	13	7	0	0	8	19
		6 hours	10,200	1	30	40	10	2	0	0	7	10
		1 day	8,200	0	28	23	20	7	0	0	8	14
		2 days	7,400	0	3	30	26	16	0	0	19	6
		3 days	4,600	1	4	23	32	6	0	1	10	23
		4 days	4,800	1	4	13	25	21	2	1	14	19
		5 days	3,200	2	3	13	23	25	1	1	8	24
		6 days	4,600	0	7	11	22	19	1	0	10	30
		7 days	6,400	1	15	24	19	21	1	0	4	19
	8 days	8,500	8	16	23	14	10	0	0	9	20	
	3 20 kilos in wt.	Before	10,400	2	30	30	11	4	0	0	4	19
		6 hours	12,400	2	25	25	13	8	2	0	15	10
		1 day	9,400	0	25	21	22	1	1	0	17	13
		2 days	9,200	0	10	23	24	10	1	0	10	22
		3 days	8,600	0	14	28	18	7	2	0	23	8
		4 days	7,600	0	7	21	25	19	5	0	15	8
		5 days	6,400	1	6	21	16	23	2	0	10	21
		6 days	7,800	1	2	18	15	26	4	0	12	22
		7 days	8,800	0	16	24	21	19	3	1	16	18
	8 days	9,800	4	19	20	14	22	2	0	14	5	
	5 14 kilos died 3d day	Before	8,200	4	30	26	21	10	0	1	5	3
		6 hours	12,600	0	35	35	8	1	0	0	8	13
		1 day	16,000	0	32	40	14	4	0	0	6	4
		2 days	4,800	1	24	36	17	6	0	0	8	8
		3 days	1,800	0	0	6	9	8	0	0	42	35
	2 11 kilos died at en d of 2d day	Before	11,000	2	15	29	15	13	0	0	8	18
		6 hours	22,000	2	30	30	16	7	0	0	10	5
		1 day	26,800	0	30	35	18	8	0	1	4	4
2 days		14,200	2	16	20	23	23	0	1	4	11	
2 c c 0.2% in 50% glycerol per kilo	4 15 kilos died 3d day	Before	6,800	3	20	23	20	6	1	0	7	20
		6 hours	10,800	4	30	30	16	8	0	0	7	5
		1 day	8,600	3	23	37	22	7	0	0	4	5
		2 days	5,400	0	5	26	25	27	0	0	7	10
		3 days	4,200	0	15	19	20	22	0	0	9	15
6 11 kilos died 2d day	6 11 kilos died 2d day	Before	15,400	1	20	31	24	17	0	0	5	2
		6 hours	25,000	0	42	33	11	5	0	0	4	5
		1 day	31,600	1	24	32	20	6	0	0	11	6
		1.3 day	25,400	2	26	32	21	11	0	0	2	6

As no results of leukocyte counts in dogs poisoned with mustard gas seem available, we insert here a table (5) giving the main results of our counts in connection with these experiments.

It is to be noted that mustard gas produces at first a decided increase in the circulating leukocytes (as high as 31,600 in a dog that died the next day); if death does not occur early the leukocytes begin to drop until a figure as low as 1,800 in dog 5 is reached just before death. In animals given a nonlethal dose, the leukocytes may drop and gradually increase again to about normal in about 8 days after injection.

The Arneth classification shows a moderate shift to the left during leukocytosis, which is followed by a shift to the right with the onset of leukopenia, reaching a point of almost total absence of leukocytes 1 and 2 as in dog 5, on the third day, shortly before death.

The mononuclear cells showed no significant change, except that in extreme cases of leukopenia there was marked relative lymphocytosis.

SUMMARY AND CONCLUSIONS

Mustard gas (dichlorethylsulphid) administered intravenously in amounts ranging from 0.005 to 0.0001 cc a few days before or coincident with the intraperitoneal injection of sheep blood to rabbits (ranging from 3 to 4 kilos in weight) had a restraining effect on the production of specific lysin and precipitin in these animals as compared to controls given sheep blood alone. The lysin curve ran about the same course as that in normal rabbits, but the latent period of the precipitin curve was much longer than normal. Given several days after the introduction of the antigen, there was still an appreciable effect of the mustard gas though less marked.

The lysin produced by dogs following the intraperitoneal injection of sheep's blood was also less after mustard gas had been given intravenously a week before and coincidentally than when given a week after the antigen administration. The agglutinin for rat blood corpuscles formed by dogs after the intravenous injection of rat blood was also lessened by the intravenous injection of mustard gas (0.5 cc, 0.1% per kilo) before or coincident with the injection of the antigen. Little, if any interference, with agglutinin production was noted if the mustard gas was given after the rat blood.

Mustard gas profoundly modifies the leukocyte count of the blood in dogs as well as in rabbits and human beings (table 5).

These experiments tend to place mustard gas in a class with the leukotoxic agents: benzene, the radioactive preparations of which thorium X is an example, and the roentgen ray, all of which, though probably differing in the details of their mechanism, have a profound effect on the hematopoietic organs, the leukocytes and specific antibody formation.

THE EFFECT OF MUSTARD GAS (DICHLORETHYL-SULPHID) ON EXPERIMENTAL TUBERCULOSIS

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There immediately arose as a result of the use of mustard gas in warfare, the question of the effect of this substance on the respiratory tract and its diseases. The universal prevalence of tuberculosis either in the latent or active form, made this question one of great importance. However, like all questions pertaining to pulmonary tuberculosis, the answer is complicated by many factors which, though capable in sum total or individually of affecting the disease indirectly by lowering the general vitality, by hastening dissemination, etc., have no direct or primary effect on tuberculosis.

Clinical reports have indicated in some cases a harmful,¹ and in others a negligible,² effect from gassing on existing and dormant pulmonary tuberculosis.

It is interesting in this connection that tuberculosis in the animal is not perceptibly influenced by agents and procedures having a profound effect on acute experimental infections. Corper and Chovey³ found that mice subjected to a single nonlethal exposure to the roentgen ray, capable however of producing a leukopenia, or given a nonfatal injection of thorium X, also capable of causing leukopenia, and shortly thereafter inoculated with pneumococci or hemolytic streptococci, human and bovine, revealed an increased susceptibility to all of these organisms, as indicated by an increased and earlier mortality among the treated animals and the earlier appearance in, and longer persistence of, the cocci in the blood, as compared with animals subjected only to inoculation. Winternitz and his co-workers⁴ found that benzene had a similar effect on pneumococcus infections in rabbits while L  wen⁵ found that prolonged roentgen-ray exposure of rabbits increased their susceptibility to staphylococcus infections, and that the resistance of mice to pyocyaneus and anthrax, and rats to anthrax was reduced by roentgenization. The tubercle bacillus, however, stands out distinctly from these organisms in respect to the leukotoxic agents as noted by Corper,⁶ Kellert⁷ and Weinberg,⁸ the course

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¹ *Riforma med.*, 1918, 34, p. 2.

² *Paris m  d.*, 1918, 8, p. 17.

³ *Jour. Infect. Dis.*, 1920, 27, p. 491.

⁴ *Jour. Exper. Med.*, 1913, 17, p. 657.

⁵ *Mittl. a. d. Grenzgebieten der. Med. u. Chir.*, 1919, 19, p. 141.

⁶ *Amer. Rev. Tuberc.*, 1918, 2, p. 587.

⁷ *Jour. Med. Res.*, 1918, 39, p. 93.

⁸ *Arch. Int. Med.*, 1920, 25, p. 565.

of tuberculosis in guinea-pigs being uninfluenced by benzene, thorium X and the roentgen ray. Hektoen and his co-workers⁹ have found that these leukotoxic agents greatly reduce the production of specific antibodies and believe that these substances may lower the anti-infectious powers of the body in at least three ways: by the reduction of antibodies, by the reduction of the number of leukocytes, and by the reduction of the phagocytic activity of the leukocytes.

In some respects mustard gas acts like other leukotoxic agents. Krumbhaar¹⁰ noted in man, especially in severe or fatal cases of gassing, that at first there is an increase in the circulating leukocytes (in individual cases as high as 36,000 and averaging in his series about 12,000), and if death does not interrupt the downward course of the leukocytes, an extreme degree of leukopenia may be reached. Krumbhaar and Krumbhaar¹¹ were able to examine the bone marrow in 55 fatal cases and conclude that mustard gas has a direct toxic action on the bone marrow affecting the hematopoietic cells. They regard the action similar to that of benzene. Pappenheimer and Vance¹² obtained similar results in rabbits following the intravenous injection of mustard gas, a marked diminution of circulating leukocytes and a specifically poisonous action on the hematopoietic tissues. Hektoen and Corper¹³ corroborated these findings in dogs given mustard gas intravenously and found this substance to have an inhibitory action on the development of specific antibodies in both rabbits and dogs.

The marked toxicity of this substance to the tissues of man and animals—the human eye may react after an exposure of less than one hour to one part in 10,000,000¹⁴ and the skin after an exposure of 30 minutes to 0.02 mg. per liter¹⁵—made it seem desirable to test the tuberculoid action of dichlorethylsulphid. Since, however, this compound is markedly unstable in aqueous solutions the problem proved to be beset with certain difficulties. Lynch, Smith and Marshall¹⁶ found that aqueous solutions would hydrolyze to the extent of 15% in 10 minutes at 10 C., while at 37.5 C. over 97% was decomposed in the same time. They found that absolute alcohol solutions of mustard gas were stable. The difficulty of working with tubercle bacilli in alcoholic solutions is obvious, so it was decided to study the effect of mustard gas in glycerol, in which it is fairly soluble, on tubercle bacilli and control the work by tests with glycerol of the same concentration as that used for dissolving the mustard gas. The following experiment may be open to criticisms, but it gives some information on the tuberculocidal action of mustard gas.

⁹ Jour. Infect. Dis., 1916, 19, p. 69; 1915, 17, p. 415; 1918, 22, p. 28; 1920, 26, p. 330.

¹⁰ Jour. Amer. Med. Assn., 1919, 72, p. 39.

¹¹ Jour. Med. Res., 1919, 40, p. 497.

¹² Jour. Exper. Med., 1920, 31, p. 72.

¹³ Jour. Infect. Dis.

¹⁴ Jour. Pharm. & Exper. Therap., 1920, 15, p. 77.

¹⁵ Ibid., 1920, 15, p. 95.

¹⁶ Ibid., 1918-19, 12, p. 265.

Actively growing cultures of human, avirulent and virulent, and bovine tubercle bacilli were well ground up in a small amount of sterile 0.9% sodium chloride solution and were then added to varying dilutions of sterile glycerol (25 and 50%) in 0.9% sodium chloride solution, and to dilutions of mustard gas in 25% glycerol ranging from 0.1 to 0.000,000,1%. The mustard gas solutions were prepared from a fresh 2% alcoholic solution of mustard gas. The suspension was well mixed, placed in the incubator for the intervals over 5 minutes and at room temperature for the shorter periods, and after a definite time a certain amount of the mixture was withdrawn, about 0.5 to 1 c c, and put into about 10 to 15 c c of sterile 0.9% sodium chloride solution, well shaken and centrifugated at high speed to sediment the tubercle bacilli, after which they were carried through the 3% sodium hydroxide procedure described by Petroff ¹⁷ and seeded on gentian violet egg medium. Two or more culture tubes were seeded for each time interval and from each dilution of glycerol and mustard gas. The results of this experiment are given in table 1.

TABLE 1
TUBERCULOCIDAL ACTION OF MUSTARD GAS (DICHLORETHYLSULPHID) FOR HUMAN AND BOVINE BACILLI

Culture	Period of Action	Controls in Glycerol		Dilutions: Percentage of Mustard Gas in 25% Glycerol in 0.9% NaCl Solution				
		25%	50%	0.1	0.01	0.001	0.00001	0.0000001
Human Avirulent	1 minute	+	+	+	+	+	+	+
	5 minutes	+	+	+	+	+	+	+
	30 minutes	+	+	+	+	+	+	+
	2 hours	+	—	+	+	+	+	+
	4 hours	+	—	—	+	+	+	+
	1 day	+	—	—	—	+	+	+
	1 week	—	—	—	—	—	—	—
Human Virulent	1 minute	+	+	+	+	+	+	+
	5 minutes	+	+	+	+	+	+	+
	30 minutes	+	—	—	+	+	+	+
	2 hours	+	—	—	—	+	+	+
	4 hours	+	—	—	—	+	+	+
	1 day	—	—	—	—	—	—	—
	1 week	—	—	—	—	—	—	—
Bovine	1 minute	+	+	+	+	+	+	+
	5 minutes	+	+	+	+	+	+	+
	30 minutes	+	+	+	+	+	+	+
	2 hours	+	+	+	+	+	+	+
	4 hours	+	—	+	+	+	+	+
	1 day	+	—	—	+	+	+	+
	1 week	—	—	—	—	—	—	—

* Two or more culture tubes were seeded at each interval from each dilution.

Table 1 reveals that dissolved in 25% glycerol mustard gas is bactericidal toward tubercle bacilli only after at least 30 minutes*

¹⁷ Jour. Exper. Med., 1915, 21, p. 38, and Corper, H. J., Fiala, L., and Kallen, Lincoln, Jour. Infect. Dis., 1918, 23, p. 267.

exposure to 0.1% and after 2 hours or more to 0.01%. These results were obtained with virulent human tubercle bacilli. They seem to be slightly more sensitive than avirulent human and bovine tubercle bacilli; the latter two cultures requiring from 4 hours' to 1 day's exposure to obtain the same effect. What part, favorable or inhibitory, the glycerol may have played remains undetermined from these experiments, although it would seem from the tests with glycerol alone that the tendency might be to enhance the action of the mustard gas on the bacilli. That mustard gas in higher concentrations dissolved in 25% glycerol will kill tubercle bacilli is brought out further by the animal experiments.

In previously reported experiments on the influence of certain agents on the resistance of guinea-pigs to tuberculosis,¹⁸ it was found that certain local irritants—turpentine, croton oil, tincture of cantharidin and tincture of capsicum—had no appreciable influence on the progress of the experimental infection in this animal, while lamp black had a distinctly retarding influence, and finely pulverized glass a markedly accelerating influence when injected with the bacilli. Krause and Willis¹⁹ recently found that the degree of allergy and immunity of guinea-pigs with an existing tuberculous focus are reduced at the site of an inflammatory tuberculin reaction for at least 4 days after the application of tuberculin. It seemed desirable, therefore, to study the local as well as the general effect of mustard gas on experimental tuberculosis, and the following experiments were devised with this in mind.

A series of guinea-pigs were infected with varying amounts of virulent human tubercle bacilli (culture Maxfield), 0.000,01, 0.000,000,1 and 0.000,000,001 mg.; 9 guinea-pigs of the series served as controls, 3 receiving the same amount of a uniform suspension of the tubercle bacilli subcutaneously into the left lower quadrant of the abdomen; 9 received the bacilli in 0.5 c c of 0.9% sodium chloride solution mixed with 0.5 c c of 0.2% mustard gas in sterile 50% glycerol, the solution being injected subcutaneously within 10 minutes after mixing; and 9 others received the bacilli in 0.5 c c of 0.9% saline solution mixed with 0.5 c c of 0.02% mustard gas in sterile 50% saline solution. Results of this experiment are given in table 2.

¹⁸ Amer. Rev. Tuberc., 1919, 3, p. 605.

¹⁹ Ibid., 1920, 4, p. 563.

TABLE 2

THE RESULTS OF THE SUBCUTANEOUS INJECTION OF MUSTARD GAS MIXED WITH A SUSPENSION OF TUBERCLE BACILLI ON THE DEVELOPMENT OF TUBERCULOSIS IN THE GUINEA-PIG

The Amount of Tubercle Bacilli Injected, Mg.	The Amount of Tuberculosis Found in the Guinea-Pig		
	Controls Given the Bacilli Alone	Animals Given the Bacilli (0.5 c c) + (0.5 c c) 0.2% Mustard Gas in 50% Glycerol	Animals Given the Bacilli (0.5 c c) + (0.5 c c) 0.02% Mustard Gas in 50% Glycerol
0.00001	++* +++ ++	-† - -†	+† ++ ++
0.0000001	+ + +	- -† -†	-† - -
0.000000001	- + +	- -† -	-† -† died

* All the guinea-pigs recorded were sectioned 5 weeks after injection. Those that died before this time are marked died. The markings are graded (from - to +++) on the basis of the amount of anatomic tuberculosis found in the guinea-pigs.

† All the guinea-pigs recorded with a † after the anatomic grading developed discharging ulcers at the site of injection, while those not thus marked did not develop ulcers.

+ = Distinctly enlarged local and slightly enlarged retroperitoneal glands.

++ = Enlarged local and retroperitoneal glands and slight involvement of spleen.

+++ = Enlarged local and retroperitoneal glands, spleen markedly involved, the peritracheal glands enlarged and the lungs slightly involved.

++++ = Massive involvement of all glands, spleen, lungs and liver.

TABLE 3

THE EFFECT OF MUSTARD GAS GIVEN SUBCUTANEOUSLY ON THE PROGRESS OF THE TUBERCULOSIS IN GUINEA-PIGS RESULTING FROM THE SUBCUTANEOUS INJECTION OF VIRULENT HUMAN TUBERCLE BACILLI

The Amount of Tubercle Bacilli Injected, Mg.	Controls Given the Bacilli Alone in 0.9% NaCl	Controls Given the Bacilli Alone in 50% Glycerol Immediately After Mixing	Guinea-Pigs Treated by a Single Subcutaneous Injection of 0.5 c c of Mustard Gas in 50% Glycerol		
			0.5%	0.2%	0.02%
0.1	++++* +++ +++ +++	+++ +++ +++ +++	All died	+++† ++ +++ Died	+++ +++ +++ +++
0.001	++ +++ ++ ++	++ ++ +++ +++	All died	+++ ++ ++ ++	++ +++ +++ +++
0.00001	++ ++ ++ ++	++ + ++ ++	All died	++ + + Died	+ ++ ++ +++
0.0000001	++ ++ ++ +	++ + + +	All died	++ + Died Died	++ ++ + ++

* See note to table 2 for the significance in anatomic tuberculous involvement of the markings - to +++++. These animals were all examined 4 weeks after infection.

† Two of each set of 4 of the animals treated with mustard gas received the injection subcutaneously in the right half of the abdomen and the other 2 subcutaneously in the region of the chest.

From this experiment it seems justifiable to conclude that tubercle bacilli in 0.1% mustard gas in 25% glycerol injected subcutaneously into guinea-pigs are not capable of causing systemic tuberculosis, likewise even 0.01% mustard gas has a distinctly retarding influence on the infection not to be explained by the sloughing out of bacilli resulting from the destructive action of the mustard gas on the animal tissues, since the same effect was noted where no ulcers developed.

In order to determine whether mustard gas given to guinea-pigs injected with tubercle bacilli had any general effect, either retarding or hastening the progress of the resulting tuberculosis, a series of guinea-pigs of uniform size—all males—were infected with different amounts of a uniform suspension²⁰ of virulent human tubercle bacilli—culture Maxfield—ranging from 0.1 to 0.000,000,1 mg. by subcutaneous injection and were then given a single subcutaneous injection of mustard gas in 50% glycerol—0.5 c c of 0.5, 0.2 or 0.02%. The results of this experiment are given in table 3.

The results obtained in this experiment indicate that mustard gas injected subcutaneously into guinea-pigs in an amount just below (0.5 c c of 0.2%) the lethal dose (0.5 c c of 0.5%) or less (0.5 c c of 0.02%), about one-tenth the lethal dose, has no appreciable influence on the tuberculosis in these animals resulting from the subcutaneous injection of different amounts (from 0.1 to 0.000,000,1 mg.) bacilli, as is determined by the anatomic distribution of the tuberculosis involvement.

In a small series of animals tested in preliminary experiments there seemed to be a retarding influence on the tuberculosis by the glycerol-mustard gas mixture given subcutaneously. Since the 50% glycerol used in this experiment had been boiled for a considerable time, it seemed desirable to repeat this experiment on a larger scale and also to test further the mustard gas (0.5 c c 0.2%) in 50% glycerol, the latter having been sterilized by only a short period of boiling or autoclaving. One set of guinea-pigs was given a single injection and another set repeated injections (3 or 4 injections spaced at 4 day intervals); likewise 50% glycerol which had been boiled for about 30 minutes to one hour, in which time a decided yellow color develops in the solution, was used for treatment and one set of the animals was given a single injection and another set 3 or 4 injections spaced at 4 day intervals. The guinea-pigs were infected by the subcutaneous injection of a uniform suspension of a culture (No. 1851) of virulent

²⁰ Jour. Infect. Dis., 1918, 23, p. 500.

human tubercle bacilli into the left lower quadrant of the abdomen. The glycerol and mustard gas was injected subcutaneously into the right half of the abdomen. The results of this experiment are given in table 4.

TABLE 4

THE EFFECT OF THE REPEATED SUBCUTANEOUS INJECTION OF NONLETHAL AMOUNTS OF MUSTARD GAS AND BOILED GLYCEROL ON TUBERCULOSIS IN THE GUINEA-PIG

Amount of Tubercle Bacilli Injected, Mg.	Controls Given the Bacilli in 0.9% NaCl	Boiled Glycerol 50% (0.5 c c) Given Subcutaneously		Mustard Gas in 50% Sterile Glycerol, 0.5 c c of 0.2% Given Subcutaneously	
		Single Injection	Repeated Injections at 4-Day Intervals for 3 or 4 Injections	Single Injection	Repeated Injections at 4-Day Intervals for 3 or 4 Injections
0.001	++++* ++++ ++++ ++++ ++++ ++++	++++ ++++ ++++ 1 died	All Died†	+++ +++ +++ ++	+++ +++ +++ 2 died
0.00001	++++ ++ ++ ++ ++++ 1 died	+++ ++ ++ 1 died	++ ++ 2 died	++ + ++ 1 died	++ ++ 2 died
0.0000001	+ + ++ + +	++ + + 1 died	+ ++ + 1 died	+ + + 1 died	All died

* The guinea-pigs in this experiment were examined 4 weeks after infection. The markings from — to +++++ designate the amount of tuberculosis found, as indicated in the footnote to table 2.

† The boiled glycerol tended to produce large ulcers which proved fatal to the guinea-pigs, especially those given repeated injections.

The experiments given in table 4 seem to indicate that even repeated subcutaneous injections (3 to 4 at 4-day intervals), consistent with the life of the guinea-pig, of a barely nonlethal dose of mustard gas in sterile 50% glycerol has no appreciable effect on the progress of the tuberculosis in these animals, as indicated by the anatomic distribution resulting from the subcutaneous injection of virulent human tubercle bacilli in amounts ranging from 0.001 to 0.000,000,1 mg. of a uniform suspension in 0.9% sodium chloride solution. Glycerol 50% in a 0.9% sodium chloride solution boiled for about one hour until it has a distinct yellow tint and given subcutaneously in amounts of 0.5 c c repeated 3 or 4 times at intervals of 4 days has likewise no appreciable effect on the anatomic distribution of the tuberculosis resulting from

the subcutaneous injection of virulent human tubercle bacilli in guinea-pigs, although glycerol has a marked local destructive action on the tissues.

SUMMARY AND CONCLUSIONS

Mustard gas dissolved in 25% glycerol is bactericidal toward virulent human tubercle bacilli after an exposure in vitro of at least one-half hour to 0.1% and after 2 hours to 0.01%. Avirulent human and bovine tubercle bacilli seem to be slightly less sensitive, these organisms requiring an exposure of from 4 hours to 1 day to obtain the same results. What part, if any, the hydrolysis of mustard gas in the glycerol played in this experiment was not determined.

Mustard gas in 0.1% strength in 25% glycerol containing virulent human tubercle bacilli and injected immediately subcutaneously into guinea-pigs entirely prevented the development of systemic tuberculosis in these animals; even 0.01% mustard gas has a distinct retarding influence on the development of tuberculosis in these animals. This action could not be due to the tissue destructive action of the mustard gas in these concentrations, since the same results were obtained in the absence of ulcers.

Mustard gas given subcutaneously to guinea-pigs in amounts consistent with life (0.001 c c in glycerol) in a single injection or repeated, at 4-day intervals for 3 or 4 injections, has no appreciable effect on the amount of anatomic tuberculosis resulting from the subcutaneous injections of virulent human tubercle bacilli.

THE FATE OF INDIA INK INJECTED INTO THE BLOOD

II. THE FORMATION OF INTRACELLULAR GRANULES AND THEIR MOVEMENTS

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The vital staining of cells is regarded by many as due to absorption of dye molecules by cell granules, but the appearance of ink granules in the interior of cells has been regarded as the result of phagocytosis. Indeed, ink granules have been introduced into the body for the purpose of testing the functional activity of cells after vital staining. Arnold¹ used dyes and other material in substance for the purpose of staining and ink granules to differentiate between the granules taken up by phagocytosis and the granules colored by vital staining, and concluded that the ink granules often were crowded closely to the cell granules and even covered the latter. Schulemann² made a similar observation and J. Koch³ injected ink into the peritoneal cavity and noted that the ink particles seemed to grow larger within the cells and seemed to be attracted to the cell granules. Evans, Schulemann and Wilborn⁴ regard vital staining as the result of a phagocytic process, but Kiyono⁵ differs from this opinion while Tschaschin⁶ observed that collargol stained a cell granule in the same way as dyes do in vital staining. Kiyono⁷ noted, however, that there was precipitation when collargol was introduced into the blood, the granules being taken up by phagocytes. Recently this investigator⁸ suggested that both vital staining and phagocytosis may be governed to a certain extent by the same physical laws but to regard them as identical processes, as Schulemann and Evans have done, is not warranted by the morphologic appearances.

No special study has been made of the migration of ink granules in the body except by Kiyono,⁹ who explains this migration as the

¹ Virch. Arch., 1899, 157, p. 424.

² Arch. f. Mik. Anat., 1911-12, 79, p. 223.

³ Ztschr. f. Hygiene und Infektionskrankh., 1911, 68, p. 80.

⁴ Deutsch. med. Wchnschr., 1914, 40, p. 1508.

⁵ Nisshin Igaku, 1914, 4, p. 917.

⁶ Folia haemat., 1913, 17, p. 317.

⁷ Folia haemat., 1914, 18, p. 149.

⁸ Kiyono: Nisshin Igaku, 1918, 8, p. 475.

⁹ Kiyono and Murakami: Kyoto Igaku Zassi, 1917, 14, p. 821.

result of excretion of granules by cells into lymph spaces and capillaries. In my work I observed that ink granules are absorbed by granules in leukocytes as well as fixed cells, which is in harmony with what J. Koch found in the case of peritoneal exudate, and furthermore I found that within the cell the ink granules become more irregular and complicated in structure than when they are first taken up.

I did not find any evidence of the exclusion of granules from cells, but I did find evidence of cell degeneration and regeneration in the course of which the granules were subjected to phagocytosis, not only a second but even a third time in such a way that eventually large accumulations would form.

OBSERVATIONS ON THE INK GRANULES WITHIN THE CELLS

In the cells the ink granules vary in size, being larger than the original granule in the ink suspension used for injection. The larger granules in the cells often are from one to one and a half mikrons in diameter. For the sake of convenience ink granules may be classified as follows:

1. The original granule, contained in the ink suspension, is a minute granule of a brownish color, too small to be measured micrometrically, with irregular margins. This granule may persist in the blood for some time.

2. The primary granule is the granule that appears in the cell first. It is usually larger than the original granule but varies much in size, and is spherical with smooth surfaces and densely black as a rule. Some granules seem a little lighter in the center and sometimes they appear unevenly colored. Granules of this kind may appear singly or form conglomerations.

3. The secondary granule is irregular and angular, often forming masses or lumps, developing as a rule some time after the injection, especially when there is a not too rapid degeneration of the cells. These granules may be associated with primary granules, the latter usually being smaller.

4. The tertiary granule is formed from the disintegration of the secondary granule and is smaller and flatter than the other granules, varies much in size and has irregular margins. This granule often looks like dust particles in the cell body or in collections of platelets.

5. By base granule I mean existing cell granules to which the original ink granules are attracted.

In order to observe these different kinds of granules best small doses of ink suspension should be injected. When larger doses are injected many cells become stuffed with granules and aggregations of granules and degeneration of cell occurs so quickly that it is difficult to distinguish various forms of granules.

As indicated, the granules appear first in the cell as primary granules, changing to secondary in a day or in a few days, this change being most noticeable in mononuclears, the polymorphonuclears often being destroyed too early. In accumulations of platelets or cellular debris the granules soon pass into the tertiary form while primary and secondary granules may and often do remain as such. In the endothelial cells of the liver, spleen and marrow the primary granule usually appears spherical and smooth soon after the injection, changing gradually into the secondary form in a few days. The accumulations of granules that eventually develop contain primary, secondary and tertiary forms, the secondary being most numerous. This mixture results from the breaking down of older granules and the new formation of primary granules. In the liver cells the granules are usually of the primary type when seen shortly after the injection, but later they are usually more irregular. In the endothelial cells of the heart and in bone cells the granules are usually of the primary form and seem to remain in this form for some time. In the surface cells and the clasmatoocyte of the omentum the granules were usually of the primary type at first, later changing to the secondary type. In the osteoclasts primary and secondary granules appeared, as well as original dustlike particles.

The question naturally arises as to how the granules of India ink injected into the body may grow so large. One might suppose that in the blood plasma the ink would not separate into the original molecules and that some at least would remain as droplets which might give rise to the appearance of a coarser granule within the cell. I have observed this after the injection of large quantities of highly concentrated ink suspension, or when I have killed an animal soon. It is a fact, however, that even the injection of small quantities of highly diluted ink gives rise to the formation of coarser granules.

To study this question more closely I injected slowly 1 c c of a much diluted ink suspension into the portal vein of a rabbit under ether. Immediately after the injection a part of the liver was removed and one hour later another part, bleeding being avoided by compression with a rubber covered forceps. Each piece was fixed immediately.

The part last removed showed larger granules in the endothelial cells, while in the part first removed there were innumerable original granules inside and outside the cells, thus showing clearly that the primary granules grow intracellularly by taking on original granules. Sections from the part first removed also showed that ink granules are attracted to the cell immediately after the injection and adhere to the surface of the cells. In the capillaries, therefore, the ink granules mark the outlines of the cell by arranging themselves around them, the so-called stellar or Kupffer cells being clearly outlined in this way. The attraction of the cells for the granules may be so strong that after injection they disappear quickly to accumulate in the liver and spleen, etc., which become intensely black while the other organs remain practically normal in color.

The reason for the growth of the granules in the cells may be explained as follows: There is in the cells concerned an element that I call the base granule around which the original ink granule becomes regularly arranged by adsorption so that the base granule becomes covered by an ink capsule, so to speak, and this in the primary form of granule. As the primary granule is constructed on the basis of the cell granule, its form will vary according to the shape of the base granule, and it is of course larger than the original ink granule and has a smooth surface. Other points of interest in this connection are that these primary granules frequently have a lighter center than border; in the incompletely developed granule the arrangement of the original granules on the base granule can be made out easily, the surface appearing roughly coated with small masses of varying hue (oil immersion and artificial light), frequently in the form of an arc or horseshoe, whereas the completely developed primary granules show a smooth black surface. When small quantities of ink suspension are injected, incompletely formed primary granules are more abundant.

As stated, the primary granule may change to the secondary form in a few days after the injection and finally into the tertiary. This tendency is more marked in the case of larger granules. The transformation seems to be connected with a change in the base granule which becomes isolated from the rest of the cell by the ink capsule and perhaps may degenerate from other causes as well, and consequently the primary granule collapses in places and finally disintegrates into the tertiary form. The smaller the primary granule the less the change in form as the base degenerates because the wall is thicker

than in the case of larger granules and also because the smaller the diameter the stronger the resistance of the wall as compared with larger granules of the same thickness of wall. This may explain why the smaller primary granules remain longer in the cells or detritus without change of form. In the liver cell proper the granules ordinarily have light centers, incomplete ones occurring, some of which may have well developed outlines. I have also noticed a sort of naked granule in liver cells in sections that were immersed longer in iodine alcohol after fixation in Zenker's fluid. Such granules stain readily with eosin. Arnold,¹⁰ by macerating cells in a solution of iodine and iodide of potassium, was able to differentiate and even to isolate cell granules (plasmosomes). As I have stated, Arnold observed that ink particles would often lie closely to the cell granule, sometimes covering them, thus suggesting that the granules are adsorbed. Examination of leukocytes in a suspension kept at 37 C. for about one hour was found to show clearly the appearances described by J. Koch, the ink granules growing larger within the leukocytes. All these facts indicate clearly that the ink granules accumulate in the cells on the base of preexisting granule-like cell elements, being built up from the small original granules, except rarely as seen in osteoclasts.

THE FATE OF THE INK LEUKOCYTE AND ITS GRANULES

As stated in my previous article, the polymorphonuclears containing ink granules become greatly diminished in number 48 hours after injection, and they disappear entirely between the fourth and seventh day, while the mononuclears though decreasing markedly, are still present. Examination of the blood reveals clearly an extensive destruction of leukocytes and the appearance of large numbers of platelets entangling much debris of tertiary ink granules; for a few days after the injection carefully made films show degenerating leukocytes as well as a remnant containing secondary or tertiary granules and also such signs of degeneration as vacuoles and loss of affinity of the nucleus for stain. In the ink cells vacuoles often seem to develop at the periphery of the ink granule, this being more obvious in the case of the secondary granules or their masses so that it may look as if the granule or mass lies on the surface or in the interior of the vacuole. The vacuole may extend and combine with others to form a large

¹⁰ Arch. f. mic. Anat., 1898, 52, p. 523.

defect in the cytoplasm and finally destroy the cell. In this way granules may drop into the blood stream from loss of support. This vacuole formation may be caused by shrinking of primary granules to secondary, but whether this is the only factor or not is questionable because they grow so extensively that the cell may break up.

The ink cells, with more of the granules, seem to degenerate more quickly than those with less. Thus, the polymorphonuclears which contain more granules decrease rapidly and in 24 hours hardly any can be found with over 10 granules, whereas in the earlier stages polymorphonuclears with more granules are frequent. Before their final disappearance from the blood stream they contain only a few granules, generally one. I never observed any tendency of the granule to fade or disappear gradually, which naturally would be expected on the assumption that they are excreted by the cells. The mononuclear ink cells, however, seem to deviate somewhat from this rule as they continue to appear in the circulation even some time after the injection, being liberated from the liver, spleen, marrow, etc. These places attract ink granules and new ink cells arise from the taking up of liberated granules from disintegrated cells, and these granules it seems are not readily taken up by polymorphonuclears. Hence we do not observe in mononuclears as orderly a decrease as in the polymorphonuclears, but that the cells which contain abundant granules degenerate more quickly than others is also indisputable in the case of these cells. Three or four days after the injection, however, especially after larger dosage, a fairly large number of cells completely filled with granules may be seen in films, indicating that at this stage numerous cells are set free.

Considering the diminution and disappearance of granules from the leukocytes, the question arises whether ink granules are excreted by cells in the same way, as many authors have observed that the dye is excreted in vital staining. As already stated in my previous paper, granules do not appear to be excreted from the body by any organ. In unicellular organisms a substance taken up within the cell body and found indigestible is expelled. It must be questioned, however, whether this mode of excretion occurs in the case of ink granules. The granule that develops in vital staining has long been a subject for discussion. Some authors regard it as a secretory granule; others consider it the result of adsorption of dye by cell elements; a few have found that stained cells may remain in the animal body

even as long as 10 months. In the case of India ink, however, it is difficult to assume that the granules are excreted in the form injected and without cell degeneration. As stated, granules may drop into the blood stream as vacuoles form, but this is the result of cell degeneration and not excretion. Not only does it seem impossible that the granules should separate again in the original form from the dense, compact, lacquer-like shell that forms but, as I have pointed out, the primary granule is converted into the secondary and tertiary kinds, the cell degenerating at the same time. Such granules have never shown any tendency to fade before their disappearance. Hence I conclude that the action of leukocytes is passive rather than active in ridding the body of granules and that the granules are not set free without degeneration or destruction of the leukocytes.

RELATION BETWEEN DESTRUCTION AND REPRODUCTION OF LEUKOCYTES

During 3 to 6 hours after injection of ink suspension, especially in large doses, destruction of polymorphonuclears and mononuclears takes place and a few hours later abnormal cells make their appearance. The abnormal polymorphonuclears that first appear in this stage have azurophil or methylenophil granules. The preexisting leukocytes, excepting perhaps lymphocytes, seem to be destroyed within 6 to 12 hours at the most and to be replaced by a new supply while still in the process of destruction. A more remarkable fact is the morphologic alteration of the polymorphonuclears into a special type, including myelocyte and metamyelocyte. The azuro- or methylenophile granules in these cells increase gradually and at 24 to 72 hours there is present a large number of such special cells with basophile cytoplasm, the size often reaching 15 to 17 mikrons and the granules 1.2 to 1.5 mikrons. It is not clear whether they are immature B granules or not; the larger always exceed the usual granules in size and are often destined in the center as undeveloped ink granules may be. They stain better when treated with concentrated Giemsa's solution for 30 to 60 minutes. The nuclei of this new type of cell also show a peculiarity in that they usually are very coarse and less lobulated, measuring often from 5 to 6 and even 8 mikrons in width. These cells at 72-96 hours, when the cell degenerating process tends to subside, appear with nicely stained nucleus and cytoplasm and decrease gradually to normal size.

Table 1 gives an example of the percentages of this large special type of cell, including myelocytes and metamyelocytes.

Thus degeneration and reproduction take place promptly. New cells are distinguishable in 6 to 12 hours and in 24 to 48 hours practically all the old polymorphonuclears are replaced by a large special type that is succeeded by a return to the normal during the next 72 hours. These special cells were observed by Pappenheim and Szecsi¹¹ after the injection of saponin and sapotoxin in rabbits, and Pappenheim distinguishes in his atlas¹² the young mother myelocytes and young mikromyelocytes, describing the latter as derived from the former and changing to the usual polymorphonuclear granulocytes through a metamyelocytic stage. The mother myelocytes appear also in chronic leukemia and in atypical acute myeloleukemia (polymorphonuclear mother myelocytes or polymorphonuclear giant leukocytes). The special large type observed corresponds with this polymorphonuclear mother myelocyte, and represents an acute regeneration of leukocytes. This shows us how many leukocytes are destroyed in a short time as that all the reservations in the marrow are sent into the circulation without time to ripen. It is of interest to note that after streptococcus injection I found that all the polymorphonuclears were replaced by this new type within 3 hours.¹³

We have no means of knowing exactly the destined length of life of leukocytes in vivo but in the case of India ink cells, especially the polymorphonuclears, it seems to vary from a few hours to 24 hours. Of course, polymorphonuclear ink cells exist in the blood even 48 hours or so after the injection, but we must bear in mind that after the degeneration of ink cells new phagocytic cells appear which take up the liberated granules. This is clearly shown by the fact that the new type, which appears mainly after the original granules have disappeared from the blood stream, also contains ink granules. The cells having basophilic granules appear as early as 6 to 12 hours, and in 24 to 48 hours they are largely changed to the special type which appears only after large doses of ink and extensive leukocytic destruction. Therefore we may conclude that after ink injection the polymorphonuclear leukocytes up to the appearance of the special new type may be replaced several times by cells of the usual type successively supplied from the reserve in the marrow.

¹¹ *Folia haemat.*, 1912, 13, p. 25.

¹² Pappenheim: *Atlas d. Menschlichen Blutzelle*, 1911-12, Supplm., p. 96.

¹³ Nagao: *J. Infect. Dis.*, 1920, 27, p. 327.

TABLE 1
PERCENTAGES OF SPECIAL LEUKOCYTES

	Time in Hours After Injection of Ink								
	6	12	24	36	48	72	96	120	
Rabbit 1, 1,450 gm., received 35 c c of ink suspension	3	12	93	...	100	89	12	0	Large cells
	97	88	7	...	0	11	88	100	Small cells
Rabbit 2, 1,350 gm., received 30 c c of ink suspension	2	8	48	72	100	65	5	0	Large cells
	98	92	52	28	0	35	95	100	Small cells

TABLE 2
INK CELLS IN BLOOD OF RABBITS INJECTED WITH BROTH AND SALT SOLUTION SUSPENSION
OF KILLED WASHED NONHEMOLYTIC STREPTOCOCCI, 15 DAYS, 3 MONTHS AND 9
MONTHS AFTER THE INJECTION OF INK

	Time in Hours after Injection of Streptococcus Suspension								
	Before	3	6	12	24	47	72	96	
Rabbit 1, 1,450 gm., received 130 c c of streptococcus sus- pension 15 days after 20 c c of ink had been injected	2	2	14	8	7	5	2	2	Mononuclear ink cells
	0	3	3.3	3	2	2	2	0.02	Polymorphonuclear ink cells
Rabbit 2, 1,950 gm., received 150 c c of streptococcus sus- pension 3 months after 30 c c of ink had been injected	1	0	2.8	6.7	1.5	0	0.5	..	Mononuclear ink cells
	0	0	2	1.4	0	0	0	..	Polymorphonuclear ink cells
Rabbit 3, 1,980 gm., received 150 c c of streptococcus sus- pension 9 months after 35 c c of ink had been injected	1	5	6	4	0	1	1	..	Mononuclear ink cells
	0	0.6	0.95	1.4	0.6	0	0	..	Polymorphonuclear ink cells

TABLE 3
INK CELLS IN THE BLOOD AFTER INTRAVENOUS INJECTION OF STREPTOCOCCUS
SUSPENSION FILTRATE

	Time in Hours after Injection of Streptococcus Suspension and Filtrate										
	Before	1	3	6	12	24	30	48	72	96	
Rabbit 1, 1,450 gm., received 5 c c of strep- tococcus suspension 7 days after 10 c c of ink suspension had been injected	1	..	1.2	3.3	-1	1.5	..	2.3	Mononuclear ink cells
	0	..	0	0.2	1	0	..	0	Polymorphonuclear ink cells
Rabbit 2, 1,850 gm., re- ceived 10 c c of strep- tococcus filtrate after 15 c c of ink suspen- sion had been injected	..	0	1	9	2	6	7.5	3.7	1.1	0.3	Mononuclear ink cells
	..	0	1	0.7	3.2	3.3	1.5	0.7	0	0	Polymorphonuclear ink cells

THE FATE OF THE ENDOTHELIAL INK CELLS OF THE LIVER,
SPLEEN, ETC.

The Degenerative Process.—The degeneration of leukocytes after ink injection is obvious and the next question is whether the endothelial cells of the liver, spleen, etc., also undergo degeneration in the same way. Appearances indicate that endothelial cells may degenerate and fall into the blood stream. In order to study the process more closely the following experiment was made: Four guinea-pigs weighing from 200 to 250 gm., each received a small dose of much diluted ink (0.3 to 1.7 salt solution) intravenously; one was killed at 24, one at 48 and one at 96 hours after the injection by bleeding and the last was killed after one month. Smears were made of the liver and spleen by pressing the slide gently against glass on the cut surface, after wiping off the blood. These smears were stained with hematoxylin and eosin, as well as the Wright stain. The endothelial cells containing ink granules showed marked signs of degeneration, containing vacuoles of various extent often occupying practically the whole cell. In fact, all fields showed many cells largely replaced by vacuoles. At 24 hours the vacuoles were small and increased gradually in size and number up to 96 hours. In this respect the changes correspond to those in mononuclear ink cells in the blood in which the most marked alterations appear 3 or 4 days after a large dose of ink. After one month, when the ink granules had become accumulated in places, the vacuoles and degenerated cells had decreased much, although still present in considerable degree. This shows clearly that the endothelial ink cells also undergo degeneration in increasing degree until the third or fourth day and that as the granules accumulate the degeneration decreases. The vacuoles described in leukocytes and endothelial cells may be secretory in the sense of Metschnikoff and others, but it may be as well a sign of cell degeneration, and the fact that ink cells break down shows that in this case it concerns a degenerative process.

THE TRANSFER OF FUNCTION

Certain authors have tried to use a combined method in vital staining by injection of two independent dyes, but the results have not been clear cut (Goldmann,¹⁴ Schulemann,¹⁵ Arnold, etc.). In such experiments there appeared usually three kinds of cells, two of which contained the granules of each color singly while the other contained

¹⁴ Vitale Karminspeicherung. Jena, 1914, p. 211; Nisshin Igaku, 1914, 4, p. 1113.

¹⁵ Ztschr. f. experiment Pathol. u. Therap., 1912, 11, p. 307.

the granules of each color. Kiyono,¹⁶ however, succeeded in producing violet granules by injection of lithium carmin and trypan blue, mixed as well as separately. Goldmann¹⁷ observed that intravital stained endothelial cells of the liver were still phagocytic with respect to India ink particles. Arnold proved leukocytes. Recently Ioka¹⁸ distinguished a kind of cell in the ovarian follicles (which he concluded to be a histiocyte) in his study of vital staining of ovary with carmin and the ink. He observed that this cell stained well with carmin and also had a strong phagocytic action on coal suspension; on the injection of a mixture of these substances both appeared in the cell; if he injected carmin first, then coal, the latter was not taken up so freely by the carmin cells; reversing the order of these injections, the results were reversed and appeared even as purely coal-pigment cells.

These results, especially the last, seem to suggest a functional limitation of ink or carmin cells, at least in some degree. To study this question the following experiment was made on seven guinea-pigs of about the same size, using ink and cinnabar suspension.

The cinnabar suspension was made by rubbing a cinnabar stick on an ink stone and filtering through filter paper without centrifugation. The suspension was sterilized by steam as in the case of India ink. The granules of cinnabar are very heavy and fall to the bottom quickly; hence, before using it is necessary to shake for some time. The granules are usually much coarser than those of India ink. The guinea-pigs were divided into 2 series, one of 4 and one of 3. The experiment was made by injecting 0.5 c.c. of each suspension (0.5 each) in a mixture or separately, adding salt solution to make the total quantity injected 2 c.c. The first group was treated as follows:

Pig 1 received both suspensions in mixture.

Pigs 2, 3 and 4: Both suspensions were injected separately, first the ink, then cinnabar, after 4 hours into pig 2, after 24 hours into pig 3 and after 96 hours into pig 4.

Each guinea-pig was killed 4 hours after the last injection and sections made of the liver and spleen.

The other group of 3 pigs was injected in the same manner as were pigs 2, 3 and 4 but in reverse order, i. e., the cinnabar first and then ink. These pigs are designated as 2', 3', 4'.

Pigs 1 and 2 gave the same result, the cells containing ink and cinnabar. Pigs 3 and 4 showed actively separate color granules; in pig 3 (24-hour interval), however, 3 kinds of granules appeared distributed in a relatively uniform manner in the cells, but in pig 4 (96-hour interval) there was a very irregular distribution of each color. The cells having many ink granules were

¹⁶ Vitale *Karminspeicherung*, Jena, 1914, p. 211; *Nisshin Igaku*, 1914, 4, p. 917 and p. 1113.

¹⁷ *Berlin klin. Wehnschr.*, 1912, 40, p. 1689.

¹⁸ *Kyoto Igaku Zassi*, 1917, 14.

generally poor cinnabar and vice versa, and there were some pure ink or pure cinnabar cells. Pigs 2', 3', 4' gave the same results. This result agrees with Ioka's and also with some of the results with vital staining, 3 kinds of cells appearing after mixed injection. In pigs 1 and 2 the granules of the cells appear to have some affinity for the cinnabar and the ink particles. I observed, however, that in these mixtures of both suspensions the ink particles tend to be absorbed on the surface of the cinnabar particles, the difference in size being quite large. Therefore the result in pig 1 may be explained in this way, but the results in pigs 2 and 2' enable one to decide definitely. We see from these results that capsulated cell granules attract or adsorb other foreign substances even 4 hours after the injection, though of course these may be influenced by the quantity of preadsorbed stuff. Later, however, this function of the base granules seems to be destroyed by the capsules of the foreign particles injected previously and the particles in the blood stream are adsorbed on other granules; but phagocytic function of the ink or cinnabar cell still remains fairly well marked. This lack of base granules is also of interest in connection with the change of the primary granules to secondary as described. After 4 days, ink or cinnabar cells, especially those stuffed with granules, are damaged greatly and lose their phagocytic action, while the cells that contain few granules still seem able to retain that function in some degree. We must, however, also consider that by this time there are newly produced cells that take up granules liberated from destroyed endothelial cells and leukocytes, and these new ink cells may take up also granules of the subsequent injection. At any rate, the phagocytic action of the endothelial cells may weaken, and on a second injection after some days the number of granules taken up may vary greatly and there may appear even pure ink or cinnabar cells, some arising from the phagocytic insufficiency of the foreign body cell, others from the activity of newly formed cells.

These experiments show that morphologically, as well as functionally, the ink cells, at least those of the leukocyte and endothelial cell nature, degenerate sooner or later after phagocytosis, and that ink or cinnabar granules are liberated again by this process of destruction, but not by secretory or excretory processes.

THE MOVEMENTS OF THE INK GRANULES WITHIN THE BODY

As described in part 1 the ink granules are not discharged from any organ, but wander from place to place until they gradually are stored up in accumulations. This wandering in the earlier stage may depend on the movements of leukocytes and logically they may be carried any place, even outside of the body, especially from foci of inflammation and from wounds or in the saliva, the sputum or other secretory fluids. But so far as I have found, the leukocytal ink cells seem to have little power to migrate, only the ones bearing a small number of granules being able to pass through the capillary walls, though when the walls are damaged they may pass out. In this sense only are the ink granules discharged from the body. This,

however, is not the real movement of the granules in the body, especially in late stages when the ink cells in the blood are few. The real transportation results from the degenerative process of the ink cells and the production of new phagocytic cells. The degeneration and destruction of phagocytic endothelial cells, especially in the early stage, not only discharges ink granules into the blood stream, but also into the lymphatics. The newly formed macrophages, including endothelial and reticular cells, now take up the granules. The appearance of granules in the later stage in connective tissue cells, perithelial cells, bone cells and in lymph nodules, etc., clearly shows the entrance of the granules into the lymph vessels, and their presence in Glisson's capsules in the liver and even in the liver cells indicate a solution of the question. The negative pressure of the thoracic duct influenced by the vena anonyma may act to draw the granules liberated from disintegrating cells into the lymph vessels through some capillary defect. In usual or lower dosage, however, any considerable quantity of granules does not pass at the same time into the lymph space; some are taken up by macrophages in the neighborhood. These new phagocytic cells may weaken and finally disintegrate, granules being sent into the stream, while new cells may be overproduced, with the result that they gradually gather here and there in groups from large accumulations of granules. This may be associated with a similar process in blood capillaries, and in advanced stages the granules may be removed almost completely from general endothelial cells, usually passing from the center to the margin of the acinus; the small accumulations also disappear gradually. In this continual replacing some granules may escape the organ through the hilus by lymph vessels or vein and circulate in general blood, being taken up by some other organ or tissue or return again to the same organ. Even more than a year after the injection the endothelial cells of liver, spleen, etc., often contain small numbers of granules and complete disappearance may not take place at any time, especially if a larger dose was introduced.

The precipitation of granules in advanced stages on the periosteum bone canals, as well as bone cells, especially the latter, situated in only a lymph space, bone lacunae, and the occurrence of ink granules evidently more in the long bones which are rich in marrow, seems to indicate that the granules do not pass into the marrow from other organs but rather pass from the marrow by way of the lymph routes from this organ. This view agrees with the decrease of granules in the marrow in late stages. In the spleen, especially in the malpighian

body as well as in the pulp, which are supposed not to have any lymphatics, the granules still formed accumulations that seem to grow while masses in other organs seem to decrease. This process consequently should proceed only by way of the blood, but we failed to observe any blood stagnation or congestion naturally expected to account for the formation of such accumulations. The accumulations also often occurred in the periphery of the central artery, as well as in the outskirts of the spleen body. That granules accumulate in the cardiac and hepatoduodenal nodes as well as in those of the neck and elsewhere is a mystery at present, but that the granules once deposited in endothelial cells in certain organs may be transferred into the lymph stream and circulate in various parts of the body is unquestionable.

The endothelial cells of the liver, spleen and marrow, which receive the granules at the same time, degenerate and some days afterward liberate the granules. At once a heavy loss of cells results, and this gives rise to compensatory phagocytosis by the endothelial cells of other organs. The greater the quantity injected, the greater the amount of phagocytosis and the quicker the destruction of cells. In such cases not only other organs compensate more freely, but newly formed cells are also excessively active. Such rapid changes should give rise to more defects of vessel walls, and all the processes should proceed more quickly than after small doses. At the time of the first injection the endothelial cells are intact and take up the granules. The stomata, if any exist, may pass such small granules easily, but the phagocytic cells hold them back. After phagocytosis of a large quantity, however, this function is lost and the granules may pass relatively abundantly through the wall, though it is necessary to bear in mind that in such large dosage as over 30 c c per rabbit some mechanical distention of the capillary walls may occur.

As stated, Kiyono also attributed this movement of granules chiefly to the lymph stream, but he believed the cells excreted the indigestible foreign body into the lymphatic spaces. I hardly agree to this point, and I would rather emphasize the degenerative processes in the ink cells as an important factor in the movement.

THE RELATION OF THE INK LEUKOCYTES AND STORED INK GRANULES

Polymorphonuclear ink cells disappear from the blood in one week after the injection. The mononuclear ink cells, however, appear

steadily in small numbers, as stated in part 1. During some days after the injection, especially when a dose is given, ink cells seem to appear in the blood by falling from the wall of capillaries containing many ink granules. Later their granule content is very small as a rule, while the endothelial cells of the liver, spleen, etc., and the splenocytes still contain considerable numbers of granules. Why the phagocytic cells do not appear abundantly in blood in the late stages is a question. The endothelial leukocyte or histiocyte (Kiyono) forms a small percentage of cells in the blood in health. Kiyono pointed out that his histiocyte as normal blood element constitutes at most 0.5% of the total leukocytes, but that succeeding injections of carmin solution may increase this number to 2%. This seems to be the case also on injection of India ink.

The problem now considered is how the endothelial ink cells (including splenocytes) behave when there is an acute demand for leukocytes, whether they fall off into the blood stream abundantly or not, and whether the cells in accumulations of many months after the injection will be destroyed.

I have an instance of a rabbit that died from some unknown cause 4 months after the injection. It showed many granules in various forms in the large vessels of various parts of the body, such as kidneys, brain, lungs, etc. This suggests that ink granules precipitated in an organ are not stable but pass into the blood freely. The rabbits were given fairly large quantities of the ink (table 2) 15 days, 3 and 9 months, respectively, before from 130-150 c c broth and salt solution in equal parts were injected peritoneally containing killed washed nonhemolytic streptococci from 24 hour growths, for the purpose of attracting leukocytes. The fluctuation of ink cells in the blood was studied before and after the injection with results given in table 2, which shows that the ink cells in the blood increased markedly until 6 to 12 hours after the injection and then decreased, returning gradually to the usual state. But the granule content of individual mononuclear cells was generally small except in rabbit 1 in which some cells were quite well filled. The most interesting thing in this experiment is the unexpected reappearance of polymorphonuclear ink cells in the blood; there appeared also free ink granules in collections of blood platelets or in cell debris. This shows that the disappearance of polymorphonuclear ink cells in late stages is not due to failure of phagocytosis but rather to disappearance of the granules from the blood, free granules in general circulating blood, if any exist, being few, most of them being taken up by phagocytes. At the same time, it is also indicated that the mononuclear ink cells which appear in blood in later stages come from fixed cells and are not of real hematogenic origin. As stated, endothelial cells or fixed macrophages with large numbers of granules failed to appear in late stages after injection (rabbits 2 and 3), while rabbit 1 showed a few. It seems, therefore, likely that these cells degenerate in loco or soon after falling off and perhaps hardly reach the blood before they liberate the granules. It is possible that a considerable number of cells may meet this fate without being observed.

The polymorphonuclear ink cells in rabbits 2 and 3 disappeared within 6 to 18 hours, while in rabbit 1, in which relatively abundant ink granules appeared free in the blood, they remained for 3 days. This also supports my view that the life of the ink cell is very short and that succeeding generations of cells, as they appear in the blood, create the impression of a longer viability. The mononuclear ink cells in the blood may originate from fixed ink cells and from preëxisting or newly formed mononuclear cells, the new cells taking up free granules in the blood.

It appears that by suitable treatment of the animals, some time after the injection, sufficient ink granules may be set free so that polymorphonuclear cells again appear in the blood as carriers of ink granules and reproduce the condition found soon after the injection of the ink. Hence it is possible that the storage of ink granules is not absolutely permanent even under normal conditions, and that the granules may be started into movement again by disturbances, such for instance as arise from bacterial infection. In this connection the results of certain experiments with the products of a nonhemolytic streptococcus are of interest. The streptococcus was grown in 1% dextrose broth for 24 hours when the culture was filtered (Maasen), and injected intravenously in rabbits previously injected with ink suspension. A mobilization of ink cells resulted, most marked about 24-30 hours after the injection, and subsiding within 72 hours. The subcutaneous injection of killed and washed streptococci did not cause the appearance of so many ink cells by far in the blood as the intravenous injection of filtrate of streptococcus cultures or the intraperitoneal injection of broth mixture, but further work will be necessary before conclusions of any value can be drawn. However, it has been shown that fixed ink cells disintegrate naturally and that their destruction may be hastened by certain disturbances and ink granules made to appear again in the blood.

DO INK CELLS MIGRATE?

A mixture of broth and salt solution was injected intraperitoneally at intervals after the intravenous injection of ink suspension, and the peritoneal fluid examined by smears 6-10 hours after the injection. At the tenth hour a quantity of fluid was withdrawn, the leukocytes collected, fixed in formol, embedded in paraffin, and examined in sections. In the smears few ink cells could be found, but in the sections ink cells were found readily, both polymorphonuclear and mononuclear, but the number of granules contained was relatively

small. This result indicates that ink cells do not migrate readily, only cells with a few granules being able to pass through.

Ink suspension alone was injected into the peritoneal cavity, also mixed with broth and salt solution, and many blood films examined 6, 12, and 24 hours later, but practically no ink cells were found in the blood, indicating that a small quantity of ink granules had reached the blood by way of the lymphatics. In the liver and spleen incompletely developed ink granules were found just as they are when minute quantities of ink suspension are injected intravenously. Goldmann, Schulemann and others demonstrated phagocytosis of vitally stained Kupffer's cells in this way in the mouse. Apparently not enough granules enter the blood to give rise to characteristic ink leukocytes, the granules being taken up by the endothelial cells in the liver, spleen and elsewhere, while most of the injected ink is taken up by the cells in the peritoneal exudate and the cells of the omentum and peritoneum and are eventually collected into masses in the omentum or elsewhere after resorption of the fluid. The cells in such masses may disintegrate and secondary, as well as tertiary ink granules, be set free and gradually pass into the blood.

THE ENTRANCE OF INK GRANULES INTO LIVER CELLS

Vital staining of the liver cells proper is difficult and requires many injections (Ribbert,¹⁹ Goldmann,²⁰ Schulemann, Kiyono, etc.). As stated, ink granules are taken up by liver cells after repeated injections. In one instance the rabbit received 4 c c of ink suspension per kilo and died 4 months later from an unknown cause; after death ink granules were found in the liver cells and also in the blood vessels in general. In 2 other rabbits injected with ink daily for 5 and 6 days, ink granules were present abundantly in the liver cells. On the other hand, healthy rabbits injected once only often did not show any ink granules in the liver cells even if the amount injected was fairly large. Kiyono appears to have had somewhat similar results. Sickly rabbits with parasitic disease of the liver nearly always showed ink granules in the liver cells even in a short time after the injection of the usual quantity. It appears therefore that the liver cells under certain conditions take up ink granules, but it is noteworthy that this does not take place uniformly throughout the liver, but only in irregular areas.

¹⁹ Ztschr. f. allg. Physiol., 1904, 4, p. 201.

²⁰ Beitrage f. klin. Chir., 1909, 64, p. 192.

According to Ogata,²¹ the lymphatics of the liver run between the columns of the liver cells and the endothelial cells of the blood capillaries into Glisson's capsule, the liver cells consequently being separated from ink granules in the blood by the endothelial layer and the lymphatic space, the former no doubt restraining them from passing into the lymphatic space. When ink granules come in contact with the liver cells they are taken up by the cells, but evidently special conditions are necessary in order to secure this contact when moderate quantities of ink are injected in the usual way. Kiyono suggests that endothelial cells may pass granules into the lymph spaces by virtue of a special excretory function, but I believe that this transfer may result rather from degeneration of endothelial ink cells. Rossle²² describes an example of phagocytosis of red corpuscles by liver cells in a case of infection of a man, and he ascribed this phagocytosis to a defect of the capillary wall rather than to diapedesis. The liver of sickly rabbits not only may show many foci to the naked eye, but also in the microscopic section the distribution of the endothelial cells may be very irregular and often groups of cells may form.

In my experiments on the distribution of streptococci in guinea-pigs after injection there was also irregularity in the distribution of the endothelial cells of the liver. In a special experiment, in which a suspension of killed and washed nonhemolytic streptococci was injected intraperitoneally and one animal killed in half an hour, another in one hour, a third after 3 hours, and the fourth after 6 hours, distinct changes in the size and form of the endothelial cells in the liver were found as early as 3 hours after the injection. In order to secure, if possible, more light on the phagocytic action of the liver cells, a special experiment was made on 3 guinea-pigs, this animal usually being free from parasites. Two of the animals were injected intraperitoneally with 70 c c of a suspension of streptococci in broth and salt solution and 20 hours later 2 c c of ink suspension were injected intravenously; one animal was killed 6 hours later, the other was given one more injection of 2 c c of ink suspension at intervals of 5 hours and killed 12 hours after the last injection. The third animal was given 4 c c of ink suspension at the same time and killed 20 hours later. In the first 2 animals the suprarenals, kidneys, lungs, and other organs were fairly black and especially in guinea-pig 2, and sections showed an extensive phagocytosis by the endothelial cells of the capillaries in general, and marked phagocytosis by the liver cells. In both cases there were many granules in Glisson's capsule. The third animal showed only normal conditions to the naked eye and no granules in the adrenal cells. The suprarenal cells of the pig injected with streptococci and once with 2 c c of ink suspension contained some ink granules in the suprarenal cells, while the pig that in addition to streptococci received 2 c c ink suspension twice showed most ink granules in the suprarenals. In both the liver and the suprarenal the distribution of the

²¹ Kyoto Igaku Zassi, 1917, 14, p. 821 (cited by Kiyono and Murakami).

²² Zeigler's Beit., 1907, 41, p. 181.

phagocytic cells was irregular; in the case of the suprarenal there was no phagocytosis on part of the nerve cells in the medulla. According to Kiyono, the cells of the suprarenal are difficult to stain with carmin, success being attained only after repeated injection. So far as I know, ink granules have not been described in suprarenal cells. The suprarenal cells in this case seem to be somewhat degenerated, hence it is possible that the phagocytosis was the result of a special condition in some way.

The fact remains that in the early stages ink granules as a rule do not pass through the capillary wall into the lymph spaces, hence it is necessary first to explain how the granules get into the lymph spaces. We know that various substances on injection may increase the flow of lymph in the thoracic duct; it is clear that if the ink granules were passed on as the result of some such mechanism their distribution would be fairly regular. As the matter stands, the irregular distribution of the ink granules in the liver suggests as the most reasonable explanation of the phagocytosis of granules by liver cells that it is the result of the entrance of granules into the lymph spaces on account of disintegration of endothelial cells and resulting defects in the capillary walls.

THE PHYSICOCHEMICAL PHENOMENA OF VITAL STAINING AND OF ACCUMULATION OF INK GRANULES IN THE CELL

Evans, Schulemann, etc., observed that the absorption of dyes by cells and the duration of vital staining are related to the physical conditions of the solution of the dye, the size of the molecules and the diffusion velocity. Dye solutions that diffuse slowly stain cells slowly but the staining may endure; on the other hand, diffusible dyes stain quickly but the staining soon disappears. Hydrosols of metals are not diffusible and stain the cells with which they come in contact more or less permanently. The taking up of India ink granules by cells has received little consideration from the physico-chemical point of view. The microscopic particles in suspension of India ink are not diffusible, and they accumulate in certain cells with which they come in contact without diffusion through the body generally; but, as I have already stated, the accumulation of the granules in cells appears to occur in the same way as in vital staining, namely, by adsorption by preexisting granules in the cells. Hence, the taking up of India ink granules by cells may be regarded as a kind of vital staining or storing up, as Kiyono states it. This view is strengthened by the fact that ink granules once taken up by a cell are not discharged except as the cell undergoes disintegra-

tion when, as pointed out previously, they may be again subjected to phagocytosis.

THE RELATION OF THE INK GRANULES TO OTHER CELL GRANULES,
ESPECIALLY THOSE CONCERNED IN VITAL STAINING

There is still much variation of opinion in regard to the nature of cell granules concerned in vital staining. Goldmann regards them as of a secretory nature; Pappenheim and Nakano²³ as plasmasomes; Loele²⁴ holds that the dye stains lipoid elements in the cells; Tschasthin regards them as in part stained chondriosomes and in part secretory; and Arnold²⁵ suggests that the granule concerned is derived from the plasmasome. The existence of different granules has been brought out by means of double stain, especially by Kiyono, who distinguishes between carminophil and trypanophil granules as well as granules with affinity for both of these stains. At present it is not clear what kind of cell elements adsorb ink granules and whether they are identical with the granules concerned in vital staining; it is a question also whether the cells that are stained vitally and ink cells undergo the same fate. At present it is clear that the ink particles are adsorbed by cell elements so as to form round capsular masses with smooth surfaces and that these masses later undergo certain changes. It is of interest in this connection to note that Anichikow,²⁶ by adding hyper- and hypotonic salt solutions, produced somewhat similar changes in the granules of vital staining. I made some experiments with the natural sepia ink of cuttle fish, an ink which consists of much smaller particles and contains tyrosin, and I found that here, especially later, the ink particles eventually form nicely arranged and regular granules in the cells. These granules apparently do not undergo any subsequent changes except a gradual loss of color, thus differing greatly from the ordinary ink granules and corresponding more to the changes described by some observers in vital staining. Other observers have noted that after repeated injections of a vital staining solution the granules in the cells will appear in various forms and that the cells may develop vacuoles and other retrogressive as well as regenerative changes. When great quantities of ink suspension are injected, many cells

²³ *Folia haemat.*, 1912, 14, p. 260.

²⁴ *Folia haemat.*, 1913, 14, p. 308.

²⁵ *Centralbl. f. Allg. Pathol. u. Path. Anat.*, 1913, 24, p. 849.

²⁶ *Nisshin Igaku*, 1914, 4, p. 917.

become so filled with ink particles that it is difficult to distinguish any definite granules one from the other, but when smaller quantities are injected the ink granules in the cells become better developed. The injection of ink and cinnabar when made at intervals was followed by the formation of pure ink granules and pure cinnabar granules in the same cell, but when ink and cinnabar were injected at the same time the granules in the cells were mostly mixed, indicating that apparently the cell element has the same affinity for these two substances. Usually the ink granules are irregular in size but in the endothelial cells of the liver they are more regular than in those of the spleen. This is true when small quantities of ink are injected; when larger quantities are injected there is more variation in the size and form of the granules in the cells, and in sickly rabbits the granules as a rule are more irregular, suggesting that there may be considerable variation in the original base element in the cells on which the ink granules form.

SUMMARY

Particles of India ink seem to be adsorbed by elements in the cells, probably granules, and a capsule of ink particles is formed which is round and smooth and which I designate as the primary ink granule. In a few days this primary granule becomes irregular, due perhaps to change of the base of the granule, and what I call the secondary ink granule is formed. As the cell disintegrates, these granules become mixed with the débris and may coalesce and form tertiary granules. The smaller primary granules seem to be more resistant than the larger and may remain unchanged for some time.

Most, if not all, cells that take up ink granules undergo destruction quite rapidly; this is associated with the formation of vacuoles, which usually appear first about the ink granules and then gradually extend. Cells that are stuffed with granules disintegrate more quickly than cells that contain only a few granules, and the life of such cells after phagocytosis would seem to last only for some hours.

At the same time that the leukocytes which take up ink granules disintegrate, new polymorphonuclears are supplied by the marrow, and in from 6 to 12 hours after the injection new cells with immature basophilic granules appear in the blood. In 24 to 48 hours later these cells are largely replaced by polymorphonuclear giant leukocytes, myelocytes, and metamyelocytes, and these cells often contain ink granules that have been set free by the destruction of phagocytes.

The phagocytic endothelial cells of the liver, spleen, and other tissues also undergo destruction from vacuolation in the course of some days, but before this time their phagocytic activity, as shown by the results of mixed or separate injections of ink and cinnabar suspensions, becomes greatly reduced. This reduction in activity appears to begin about 24 hours or so after the first injection of ink because after that time new granules often do not appear to be formed in the phagocytic cells.

The ink granules within the phagocytes are set free by the destruction of the phagocytes and not excreted by special cell function. Naturally, phagocytic leukocytes transport granules to some extent and may carry them to the outside of the body, but the main movement of the ink granule is by way of the blood and lymph stream acting on granules freed from disintegrated cells, and such granules may be taken up by phagocytic cells with which they come in contact. Repetition of this process of cell disintegration, liberation of granules, and phagocytosis by new cells eventually results in irregular accumulations of ink masses.

When leukocytes are induced to enter the peritoneal cavity at different stages after the injection of ink, there may again appear in the blood polymorphonuclear and mononuclear ink cells. The latter are probably not merely cells that have fallen off from the endothelial lining or that have been set free from cell accumulation, but come also from mononuclear cells in the blood that are engaged in secondary phagocytosis. The intravenous injection of products of streptococci produce the same result in some degree, but subcutaneous injection of such products has only a slight effect. These results indicate that ink granules may be set in motion again by various procedures, particularly perhaps by bacterial infection.

The injection of chemotactic solution into the peritoneal cavity results in the attraction of cells with few ink granules only; apparently phagocytes stuffed with granules are not able to migrate, and the ink leukocytes that form in the peritoneal cavity do not seem to be able to return to the blood. The ink granules are transferred in small degree only to the blood by way of the lymph in the earlier stages and form primary granules in the liver, spleen, etc.

After such treatment the phagocytosis of ink granules by the endothelial cells of the liver and the suprarenal was followed by the appearance of ink granules in the cell proper of those organs and also in the case of the liver in Glisson's capsule, but the distribution of

these granules was very irregular, suggesting that through some defect of the capillary wall the original ink granule passed easily into the lymph spaces.

The mechanism of the accumulation of ink granules in the interior of cells would seem to be similar to that of vital staining by soluble dye, being governed largely by physical conditions. The granules being nondiffusible, are not discharged from the cells by any excretory process, but liberated only on disintegration of the cell. The base granules which adsorb ink granules appear to be produced in large numbers by the cells after the injection so that the cell becomes filled with ink granules, but in such cases the newly produced granules seem to be more irregular than those present before the injection.

PLATE I

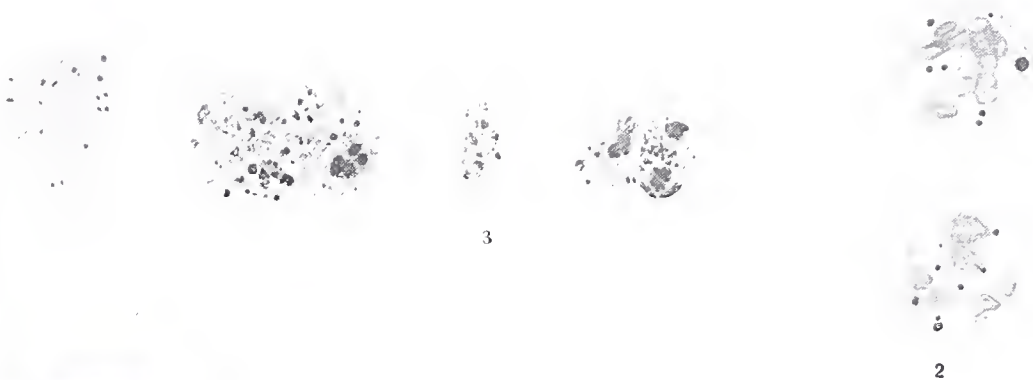
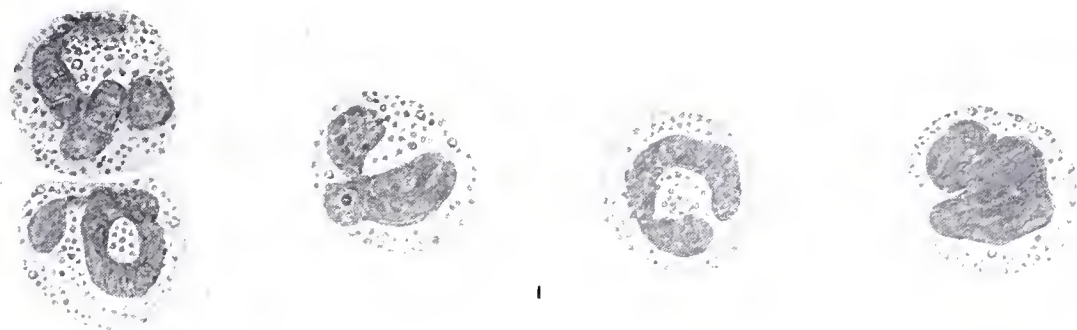


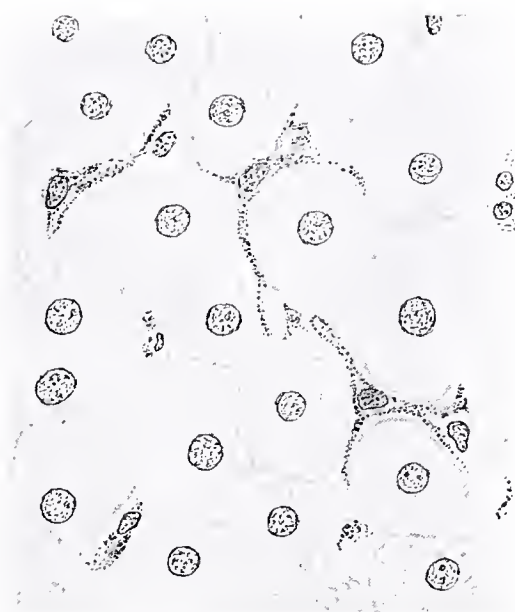
Fig. 1.—Various kinds of polymorphonuclear giant leukocytes with basophilic granules in cytoplasm. Ocular Leitz 4; Spencer oil imm. 1.8.

Fig. 2.—Usual type of polymorphonuclear leukocytes containing mostly primary granules.

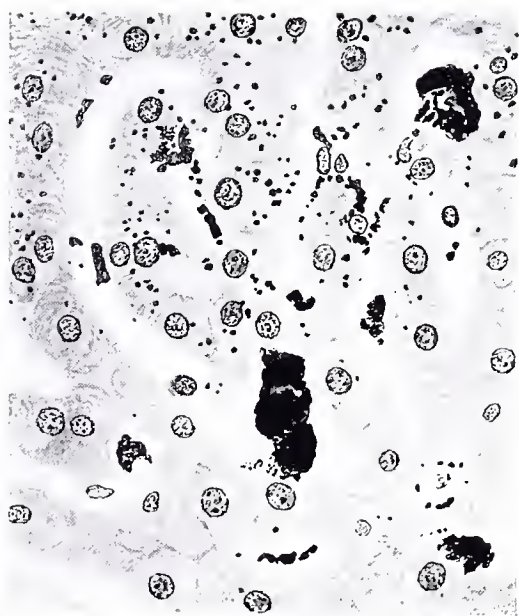
Fig. 3.—Various kinds of ink granules, especial tertiary, in debris of cells and accumulation of platelets.

Fig. 4.—Mononuclear leukocytes with various forms of granules showing vacuoles and cell destruction.

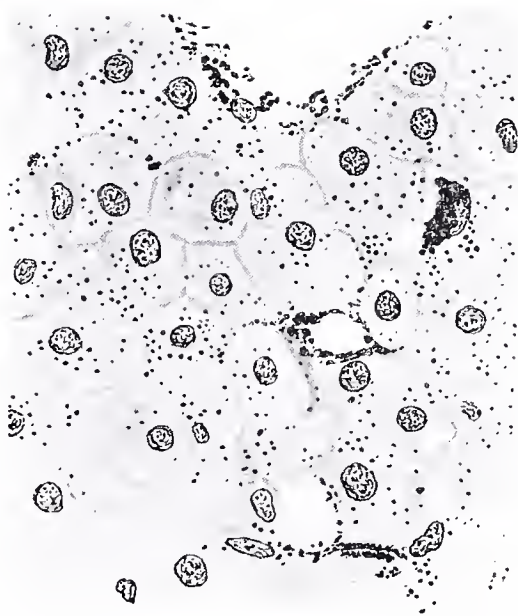
PLATE II



5



6



7

Fig. 5.—Endothelial cells in the liver of a rabbit immediately after the injection of a small quantity of dilute ink into the portal vein. Leitz ocular 6; Spencer objective 1.8.

Fig. 6.—Phagocytosis by liver cells in a rabbit with coccidiosis. Leitz ocular 2; Spencer immersion objective 1.8.

Fig. 7.—Phagocytosis by suprarenal cells of guinea-pig which had received 2 c.c. of ink suspension twice following intra-abdominal injection of killed streptococci. Same magnification as Fig. 6.

AVIAN BOTULISM (TYPE A) OR LIMBER NECK

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A sporadic toxemic syndrome in chickens, or a closely allied disease of the barnyard fowl, has been recognized for many years in the urban and rural poultry flocks of America. Affected birds display such prominent symptoms as weakness, incoordination of movement, prostration, coma, and inappetence, while the striking feature of the disease is a weak, pendulant, or limber neck. The latter, easily recognized, symptom has been popularly designated as the name of the disease.

Until Dickson¹ suggested that *B. botulinus* might be an etiologic factor in so-called limber neck, little was conjectured relative to the cause or causes of this disease in poultry. In his investigation of a case of botulism in man, a fatal illness in chickens developed simultaneously. The clinical manifestations in the fatally afflicted chickens were repeatedly induced by feeding portions of contaminated human food, and *B. botulinus* was isolated from the alimentary tract of affected birds. Since this observation, investigators have expressed a deep interest in the etiology of limber neck. The toxic effect of *B. botulinus* when fed to chickens has been sought as a contributing or supporting factor of Dickson's original contention, but the results in the hands of independent investigators are not in full agreement.

TOXIC CHARACTER OF *B. BOTULINUS* FOR CHICKENS DEPENDENT ON TYPE OF TOXIN

It is even possible that the symptoms characteristic of limber neck may involve a group of diseases induced by a variety of agents, yet it seems well established from the evidence at hand that one type of the syndrome under discussion may be induced by *B. botulinus* type A; while *B. botulinus* type B as yet has not been found associated with the sporadic poultry disease in question. Moreover, in repeated trials it has not been possible to produce illness in chickens by feeding liberal amounts of *B. botulinus* type B disguised in wholesome feed.

The numerous discrepancies reported relative to the toxicity of *B. botulinus* for chickens is responsible for the opinion that this character

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¹ Monograph No. 8, Rockefeller Inst. for Med. Research, 1918.

may not be an attribute of all strains of the organisms or that the susceptibility of the exposed birds or the quantity of toxin consumed may be an important or deciding factor. While such relative factors cannot be entirely excluded, the incidence of illness in chickens following the feeding of the proper amount of specific botulinus toxin apparently depends on the type of the toxin employed, in accord with the suggested biochemic classification of Burke. The elimination of misunderstanding or confusion on this point will, therefore, doubtless imply more attention to a specific designation of the strain of *B. botulinus* employed. Recognition of each strain of *B. botulinus*, referred to in literature in accord with the present arbitrary classification as types A and B, is dependent, in our opinion, on the expressed toxicity of the strain for chickens.

It is not intended to refute the possible relation of the many specific or nonspecific factors that have been mentioned by investigators, clinicians and poultrymen in connection with this disease in poultry, but to restrict and qualify, if possible, in accord with our findings, the seeming etiologic relation existing between limber neck in chickens and avian botulism with *B. botulinus* type A. The outbreaks of limber neck in poultry coming to our attention have not been associated with illness in the human family as reported by Dickson. Nor have the losses, to our knowledge, occurred on such an extensive scale as reported by Hart,² yet the economic importance of diseases of poultry similar to limber neck has been repeatedly suggested by the mortality in several small flocks.

CHICKENS REFRACTORY TO *B. BOTULINUS* TYPE B

Following the report by Dickson³ that chickens were susceptible to *B. botulinus* and that the clinical manifestation resembled so-called limber neck, Buckley and Shippen⁴ fed chickens *B. botulinus* isolated from cheese without producing noticeable symptoms, while Wilkins and Dutcher⁵ concluded, "It was not possible to produce limber neck symptoms in poultry by feeding and injecting the toxins produced by three different strains of *B. botulinus*. The strains were toxic, however, for guinea-pigs." Previous to either report, Van Ermengem and Roemer found that chickens were not susceptible to certain strains of *B. botulinus*. We repeatedly attempted to determine the toxicity of botulinus

² Jour. Amer. Vet. Med. Assn., 1920, 10, p. 75.

³ Ibid., 1917, 3, p. 612.

⁴ Ibid., 1917, 3, p. 809.

⁵ Ibid., 1920, 10, p. 653.

toxin to chickens by feeding liberal amounts of the toxin. From 2 to 4 mature chickens were included in each lot. The toxin was carefully mixed in the feed or given from a capillary pipet. Only negative results were obtained. Since these first results were reported,⁶ eight strains of *B. botulinus*, all of which later proved to be type B, isolated from remote outbreaks in food poisoning in domestic animals, have been fed to mature chickens. Symptoms of illness could not be detected at any time in these fowls. The negative results reported in feeding *B. botulinus* to chickens suggest, in the light of our present knowledge, that other investigators also employed type B strains in their experiments.

THE RELATION OF CHICKENS OR OTHER RESISTANT SPECIES
TO THE EPIZOOLOGY OF BOTULISM

Although the barnyard fowl and other animals may possess a natural tolerance to one or the other type of botulinus toxin, it cannot safely be assumed that they may not be an important factor in the distribution of the virus. Mature swine have displayed a marked resistance to *B. botulinus* type A, while under certain conditions mature cattle have apparently possessed a marked tolerance, though the virus could be found in the excreta of these animals. The latter observations have been repeated when unfiltered broth cultures of *B. botulinus* have been fed to hyperimmune horses, i. e., the feces have been found to contain the spores of the organism. Buckley and Shippen⁷ observed that chickens fed *B. botulinus* toxin remained healthy, yet the feces of the fowls proved poisonous to horses when disguised in their feed. In one instance poisonous chicken feces were found in an oat hay⁶ which had been given horses, among which a sporadic outbreak of botulism had occurred. It is significant that the farm poultry on the premise remained healthy, and that these birds had limited access to other feeds stored in the barn. The relation of the presence of *B. botulinus* (later proved to be type B) to the chicken excreta in the feed in question was not definitely established, yet it was suggested that one of the following possibilities might be true:

- (a) The chicken excreta had primarily contaminated the oat hay.
- (b) The chickens had eaten of the contaminated oat hay and the virus had passed through the avian alimentary canal to the forage unaltered, or if altered en route, had regained its virulence.

⁶ Graham, Robert; Brueckner, A. L., and Pontius, R. L.: Ky. Agric. Exper. Sta. Bull. 207, 1917, p. 60.

⁷ Jour. Amer. Vet. Med. Assn., 1917, 3, p. 809.

(c) The chicken excreta had been discharged uncontaminated with *B. botulinus*, and by direct contact with the unwholesome oat hay had become contaminated with *B. botulinus*.

B. BOTULINUS TYPE A FATAL TO CHICKENS

The toxic effect of *B. botulinus* type A on chickens was first observed in feeding a strain isolated from olives. Typical symptoms of limber neck could be repeatedly induced by adding the toxin to the feed or by giving it with a capillary pipet. The striking variation in the pathogenicity of different strains of *B. botulinus* to chickens suggested the importance of classifying the toxins according to the method of Burke.⁸ All strains that failed to induce illness in chickens were found to be *B. botulinus* type B, while two strains from olives, one strain associated with a fatal pasture disease of horses, one strain from the feed of swine associated with the death of nursing pigs, one strain from the spleen of a calf, as well as other strains from outbreaks of limber neck in poultry, which on being fed to chickens produced fatal illness indistinguishable from limber neck, proved to be *B. botulinus* type A. The symptoms may be observed from 5 to 6 hours after the feeding of the toxin and death may follow in 24 to 48 hours.

The evidence strongly suggests that an untyped strain of *B. botulinus* may be readily and accurately recognized by feeding the toxin to chickens. As an independent, confirming or preliminary method of identifying or typing *B. botulinus*, it may be employed to expedite a diagnosis without incurring the delay involved in isolating and propagating the specific anaerobe, which may involve several days or weeks. This preliminary determination may be accurately accomplished if unaltered contaminated food is available for feeding purposes. Furthermore, should a careful survey in outbreaks of botulism in domestic animals reveal the remote or even simultaneous occurrence of limber neck in chickens, the writers would feel justified in recommending specific *B. botulinus* antitoxin type A. In fact, our experience leads us to believe that the latter antitoxin is indicated in the treatment of this disease in poultry in preference to the polyvalent variety.

AVIAN BOTULISM TYPE A

The concrete relation of *B. botulinus* Type A to the sporadic disease in poultry designated limber neck is briefly suggested in the bacteriologic findings of the following typical outbreak.

⁸ Jour. Bacteriol., 1919, 4, p. 555.

Specimens 823 to 829, delivered to the laboratory Oct. 22, 1920, consisted of 6 full-grown chickens. The birds were prostrate and unable to stand. According to the owner, the flock, which consisted of 70 birds, was apparently in good health until 3 days before. On this date one bird was found dead. The following day 2 more dead birds were found in the yard. The third day, and at the time the specimens were delivered, between 10 and 15 birds were visibly ill. On the same afternoon a survey of the premise and a careful inquiry relative to the feed given, revealed the fact that no grain was regularly fed to the flock. A limited amount of corn was probably obtained by the birds from a nearby corn field, as well as corn fodder stacked near the barn. The latter appeared wholesome and was being fed to a cow. At least a portion of the ration consumed by the chickens consisted of freshly gathered restaurant garbage which the birds obtained from the feeding floor of an adjoining pig lot. We gathered samples of this refuse from the feeding floor in sterile vials for examination. The pigs consuming the garbage remained healthy, yet the mortality in the fowls approximated 80%, with the losses distributed over a period of two weeks.

B. BOTULINUS TYPE A ENCOUNTERED IN GARBAGE AND INTESTINAL CONTENTS OF DEAD CHICKENS

Omitting the details of the examination, *B. botulinus* type A was encountered in the composite sample from the intestinal tract of several of the dead birds, as well as from a composite sample of the garbage of the feeding floor. The two strains isolated proved analogous and capable of inducing the syndrome of limber neck in chickens. The result of the typing test is shown in table 1.

TABLE 1
RESULTS IN TYPING TEST

Number	Weight of Chickens, Gm.	Treatment* Nov. 5, 1920, 10 a. m.	Results
424	250	100 L. D.† <i>B. botulinus</i> , strain 829, 10 units type A antitoxin.....	Remained healthy
449	260	100 L. D. <i>B. botulinus</i> , strain 829, 10 units type B antitoxin.....	
428	250	100 L. D. <i>B. botulinus</i> , no antitoxin.....	Dead, Nov. 6, 1920, 7 a. m.

* Toxin administered via the mouth with capillary pipet; antitoxin injected simultaneously beneath the skin.

† Guinea-pig lethal dose.

The A type antitoxin used in this test was a sample from Dr. George A. Hart, Veterinary Department, University of California. The history of the strain from which the antitoxin was produced is not known to us, yet the serologic relation to avian type A, strain 829, is clearly suggested.

BOTULINUS ANTITOXIN TYPE A, A SPECIFIC PROPHYLACTIC

Limber neck in chickens induced by *B. botulinus* type A may be prevented in healthy fowls by injections of 50 units, more or less, of specific type A antitoxin. The results of immunologic tests in chickens are suggestive of the value of antitoxin in the control of this condition in natural outbreaks. The curative value of the antitoxin in chickens

is unknown, since the few trials to which it has been subjected to date have not permitted definite deductions. As a prophylactic it has not, as yet, been used extensively under field conditions, but in the laboratory it has been repeatedly observed that 50 units of antitoxin are quite sufficient to protect mature chickens against 800 lethal doses of toxin for guinea-pigs. In fact, the latter amount of toxin may be repeated on three or four consecutive days without producing noticeable illness, if preceded by a subcutaneous injection of antitoxin.

The type B antitoxin is not in our observation indicated in the treatment of limber neck. Its failure to control the disease as induced by type A is shown in Table 2.

TABLE 2
RESULTS OF USE OF TYPES A AND B ANTITOXIN

Number	Weight Gm.	Toxin-Antitoxin Dec.20, 1920, 10 a. m.	Results
869	1,430	829/800 L. D. 20 u. A.	Remained healthy
870	1,460	829/800 L. D. 40 u. A.	Remained healthy
871	1,570	829/800 L. D. 80 u. A.	Remained healthy
872	1,640	829/800 L. D. 160 u. A.	Remained healthy
873	1,600	829/800 L. D. 100 u. B.	Died, Dec. 21, 1920, 2 p. m.
874	1,830	829/800 L. D. control.....	Died, Dec. 21, 1920, 2 p. m.

CONCLUSIONS

The difference observed in the toxicity for chickens of the various strains of *B. botulinus* type A and type B, as well as the primary relation of *B. botulinus* type A to limber neck in poultry, suggests the following conclusions:

B. botulinus type A, via the digestive tract, is fatal to mature chickens. The symptoms induced are indistinguishable from so-called limber neck. *B. botulinus* type A has been isolated from the feed and the intestinal content of birds fatally afflicted in sporadic outbreaks of so-called limber neck. Stated another way, at least one type of limber neck occurring in poultry in Illinois is associated with *B. botulinus* type A.

B. botulinus type B, via the digestive tract, is nontoxic to mature chickens, yet the feces of chickens fed this strain are highly toxic to susceptible animals. That the virus may be distributed in nature by apparently healthy chickens or other resistant animal species is suggested.

Botulinus antitoxin type A is a specific prophylactic agent for botulism in chickens, and may be conservatively recommended in the prevention and treatment of outbreaks of so-called limber neck in poultry induced by *B. botulinus* type A.

DIPHTHERIA CARRIERS AND THEIR TREATMENT WITH MERCUROCHROME

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Diphtheria being a preventable disease, the sudden outbreak of three cases in as many days in the Lock Ward of the U. S. Naval Hospital, Mare Island, Calif., caused the following steps to be taken to check its spread. All patients, personnel and civilian employes in the hospital compound were immediately examined and Schick tests, nose and throat cultures were made. Of 544 Schick tests, 104, or 19.1%, were found to be positive. These susceptible men were immediately given 1000 units of diphtheria antitoxin and a course of three injections of toxin-antitoxin was commenced. All new patients brought to the hospital were first taken to the laboratory where cultures were made and where they received the Schick test before they were admitted to their wards. Regarding the cultures made of those in the hospital compound, special care was taken to enter thoroughly the nasal passages and to extend the swab well back to the nasopharynx. In the throat an attempt was made to have the swab enter the tonsillar crypts and the pharynx. The cultures were then grown on Loeffler's medium, and the smears stained by Neisser's method and examined carefully.

A rather high percentage of carriers of diphtheria bacilli were found, 162 of 680 persons examined being positive. The majority of positive reactions were secured from the nasal passages, and we believe were in no small part due to the thoroughness used in making the cultures, by having the swabs come in contact with all parts of the mucous membranes. Our percentage of 23.8 is considerably in excess of the 2.76% found in 3,215 persons examined at Camp Sherman, Ohio, in 1918.¹ It conforms, however, fairly closely with the figures of Labit of the French army, who obtained 28.9%, and at times 50%, of carriers in the environment of diphtheria at a military hospital.² We consider that there is an environment of diphtheria at this station as the disease is practically endemic in the nearby towns.

This large number of carriers was so distributed throughout the various hospital wards and personnel that to have isolated them all

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¹ McCord, Friedlander and Walker: Jour. Am. Med. Assn., 1918, 71, p. 275.

² Arch. de méd. et de pharm. mil., 1917, 67, p. 779.

would have seriously interfered with the functioning of the hospital. As every one connected with the hospital was immunized against diphtheria, it was decided not to isolate the carriers but rather to attempt to make them carrier free just as soon as possible, by conducting them through a thorough and systematic course of treatment. In looking over the various methods of treating carriers the results were rather discouraging, and there seemed to be no particular method of choice other than to use some germicide and to apply it thoroughly and systematically to the mucous membranes of the nose and throat. We therefore decided that the first rule governing our treatment of the carriers would be to enforce a consistent, thorough and systematic application of whatever germicide or agent we chose to use, to the walls of the nose and throat. In this way we hoped to have the germicide reach, as far as was in our power, the hiding place of the organisms. The problem of treating the carriers was placed in the hands of the laboratory staff, who had the facilities for making the necessary cultures at intervals, to check up on the treatment. All ambulatory carriers were therefore to report to the laboratory for treatment at 8:00 a. m. and 5:00 p. m. daily, while bed carriers were to be treated in their wards by a representative of the laboratory. The minimum standard set, before a patient was considered as being carrier free, was three consecutive negative cultures taken at 48-hour intervals, the third culture being secured at least 24 hours after the last local treatment. The cultures were made in the morning before treatment.

In searching for a germicide, we had to consider the enemy we were engaged against, knowing full well his predilection for hiding places in crypts of the tonsil, between the folds of the mucous membranes, in erosions, ulcers and also behind any shelter he can find in the nasal and pharyngeal passages. As a matter of fact, we considered that we were facing a situation somewhat similar to the one that the genito-urinologist is confronted with, in trying to have his antiseptic solutions reach the gonococcus, as it establishes itself beneath the cells and in the glands, lining the mucous membrane of the urethra. The report by Young, White and Swartz,³ therefore, on the uses of the new germicide mercurochrom-220 (a bisodium salt of dibrom-oxymercury-fluorescein), lauding the marked penetrating and germicidal properties of that drug, and demonstrating that it can be utilized in relatively concentrated solutions on the mucous membranes of the bladder and urethra

³ Jour. Am. Med. Assn., 1919, 78, p. 1483.

without causing irritation, gave us the suggestion to use this same drug germicide in an attempt to reach the diphtheria bacilli in their various hiding places in the nose and throat. We, therefore, used aqueous solutions of mercurochrome in $\frac{1}{2}$, 1 and 2% strengths.

Of our total number of carriers we were able to treat, consecutively, thoroughly and systematically, only 90. The remaining were treated more or less itinerantly, and have not been included in our figures regarding the efficacy of mercurochrome in the treatment of diphtheria carriers. One or two of the medical officers, some of the nurses and several of the patients obtained the idea that our procedure was to be more or less of an experiment, and we encountered some lack of cooperation in the use of this germicide. As a result, therefore, for those who objected to receiving the mercurochrome, we used any one of a number of substances, such as diphtheria antitoxin, argyrol, normal salt solution, etc.; and as they appeared only at irregular intervals, we obtained results no different from those usually obtained in treating carriers by the old and more or less established procedures, as noted later. Then, too, 16 of our carriers were discharged to duty from the hospital. Five others absconded, and 8 more were found to be in the civilian Chinese help, and it was next to impossible to give them any kind of systematic treatment. They were treated whenever we could reach them. This leaves 51 of our carriers who were not treated systematically with mercurochrome. Of this number, we are still treating 35 with various antiseptics as mentioned and 16 have gone to duty as being carrier free. This treatment has been going on for about 35 days so that by the time the condition of our 35 remaining carriers clears up, they will have a number of sick days far in excess of the average number for the carriers treated with mercurochrome.

Eighty-eight of our 90 carriers treated with mercurochrome were made carrier free with an average of only 19.1 treatments, or applications of the germicide, with an average of but 12.7 sick days. The 2 extra sick days (19.1 treatments approximating 10 days) resulted from one day in the hospital without treatment before his last culture had been made, and another day passing before we were able to obtain the result of this final culture. The remaining 2 of the 90 carriers resisted all our efforts as they continued to harbor the bacilli in tonsillar crypts and in the scars of a peritonsillar abscess. One of these two was even treated by having him lie on his side, flooding the tonsillar area with a 2% solution of mercurochrome, and retaining it there for 10

or 15 minutes, but without avail. We finally had to resort to tonsillectomy in this case. This patient's nose, which was also positive (a clinical case), cleared up relatively early under the use of mercurochrome, but the throat, although we obtained an occasional negative culture, consistently remained positive.

In not over 10 persons treated with mercurochrome solution was there any complaint as to the germicide being irritating. Several of these persons who were sensitive to the drug were medical officers and nurses. They described it as a feeling of congestion in the nose. One nurse complained of a marked dryness of the mucous membranes of the nasal and pharyngeal passages, but we discovered that she had the same sensation from the application of antitoxin and other antiseptics. In the latter case, we resorted to normal salt solution, and she was speedily relieved of symptoms as well as of diphtheria bacilli.

The 1% solution of mercurochrome was used as a routine application by means of a medicine dropper, spray or swab. In the more resistant cases we used the 2% strength, and in cases showing any evidences of congestion or irritation, we used the 0.5% strength. When persons complained of the application of the drug by means of the nasal swab, we used the medicine dropper and had them hold their heads back for a minute or two after the application, until the drug passed back into the nasopharynx. We relied on the medicine dropper in the nasal tract almost entirely and on the swab for the tonsils. In patients with nasal obstructions we utilized the nasal spray as a means to have the germicide reach the organisms.

CONCLUSIONS

Our high percentage of diphtheria carriers conforms to those of Labit of the French army for men in a diphtheria environment.

In addition to systematizing the treatment of diphtheria carriers, we have found that the germicide of choice is a solution of mercurochrome-220 in 0.5, 1 or 2% strength. By using this drug we were able to obtain in our series of carriers of diphtheria bacilli 88 carrier-free persons, following an average of 19.1 applications of this solution, with an average of only 12.7 sick days, as compared to an average of 23 days, the best results we have found recorded for a large number of carriers.

DIPHTHERIA INFECTIONS, WITH PARTICULAR REFERENCE TO CARRIERS AND TO WOUND INFECTIONS WITH *B. DIPHTHERIAE*

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AND

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Seventy-five cases of diphtheria and 102 diphtheria carriers were treated at the Walter Reed General Hospital during the period from Oct., 1918, to Aug., 1919. Thirty-seven of the infections developed in persons who were living in the hospital either as enlisted attendants or as patients admitted for other conditions.

Theoretically it should be possible to stop the spread of diphtheria in institutions, either by the isolation of all cases and carriers; or by the immunization of all nonimmune persons. The former method, which is the one generally used, in both civil and military hospitals, was carried out with rather poor results. Immediately following the bacteriologic diagnosis of a diphtheria case, or carrier, the patient was transferred to the proper isolation ward. Throat cultures and Schick tests were made of all men who had been in contact with him, and when indicated, these contacts were immunized with diphtheria toxin-anti-toxin mixture. All cultures were saved and an effort was made to determine the virulence for guinea-pigs and the special cultural characteristics of each.

A direct relationship apparently existed in a number of instances, between carriers and clinical cases. Twelve cases and 14 carriers were found to have been in contact with A——, a nasal carrier, who had been a waiter in the main mess hall. This man gave no history of diphtheria but had suffered with a chronic rhinitis for five years. Cultures of both nares continued to yield diphtheria bacilli mixed with *pyocyanus* in spite of treatment, for months. The importance of this case is doubtful as the diphtheria bacilli isolated were avirulent for guinea-pigs. However, as little is known concerning the possible changes in virulence following the transfer of organisms from one

human being to another, it is mentioned. In one small group of surgical patients who habitually played cards together, three cases developed and four carriers were found. On Feb. 15 four carriers were found among the members of a band that had been transferred as a unit from Camp Greenleaf. Diphtheria bacilli were also obtained from the mouthpieces of musical instruments used by these men. M——, who carried virulent bacilli, was treated for 8 weeks and returned to duty after the required number of negative cultures. Two weeks later another musician, who slept in the bed next to his, developed a severe case of laryngeal diphtheria. Cultures made at this time of both men contained the same type of virulent diphtheria bacilli as those originally obtained from M——.

GROUPING OF CASES

The cases considered here have been divided into three groups as follows: (1) throat carriers; (2) wound carriers, and (3) active wound cases.

The first group has been subdivided into (a) convalescent carriers, or those who have had active diphtheria and who harbor diphtheria bacilli in their throats during convalescence; and (b) contact carriers, who, as the name implies, became carriers through contact with other carriers or active cases of diphtheria, without having the disease clinically.

The term wound carrier is applied to patients whose wounds contained diphtheria bacilli, but showed no signs of an active diphtheric process. Organisms were isolated from amputation stumps, thoracotomy wounds, shrapnel and other flesh wounds and bone sinuses. All of these wounds showed granulation tissue which was apparently healthy. The presence of the diphtheria bacilli did not seem to prevent or delay healing. Positive cultures were obtained in a number of instances up to the time when the wounds had completely healed.

Into group 3 have been placed all active wound cases showing a membrane with accompanying inflammatory changes. The membranes were usually whitish, gray or grayish-green in color, with raised, well defined edges. They were tightly adherent, and on being stripped left bleeding surfaces. In two cases the membranes extended over the edges of the wounds on to the surrounding skin. Pain and tenderness were marked symptoms in all of these cases. Wound carriers, on the other hand, rarely complained of tenderness and never of pain in their

wounds. General reactions of the cases varied. In the severe ones the temperature remained around 102 and 103 F., while the pulse rate was from 100 to 120. In others, however, there was only a slight elevation of the temperature and pulse rates, and the patients complained only of painful wounds; in fact, some of them objected to staying in bed.

Routine cultures of the nose and the throat were made of all patients admitted to the isolation wards and three successive negative cultures, made at three-day intervals, were required before their release. When wounds were present cultures were made from them. The guinea-pig virulence of the organisms from each case was determined as soon as possible, and carriers of virulent organisms were separated from carriers of avirulent organisms. Schick tests were made on most of the patients, and all those showing a positive reaction were given three 1 c.c. injections of diphtheria toxin-antitoxin mixture at 7-day intervals.

TREATMENT

Patients with active cases of diphtheria, it need scarcely be said, were given antitoxin during the acute stage. When convalescent they received the same treatment as that given contact carriers, which consisted of local applications of various antiseptic solutions and in selected cases, surgical removal of infected foci. In the treatment of many of the cases Dobell's solution was used as a gargle, and as a nasal and pharyngeal spray, at 8 a. m. and 4 p. m. each day, with liquor antisepticus alkalinus in the same manner at noon and at 8 p. m. Cultures were made from the nose and throat of all carriers, after the second week in isolation; and from the crypts of the tonsils, after thoroughly scrubbing the tonsillar surface with antiseptics, such as dichloramine-T, phenol, 5%, in glycerol, or Dakin's solution. Those showing positive crypt and negative nasal cultures, were transferred for tonsillectomy. The treatment of the patient whose history is given in the following, who on admission had positive nasal, tonsillar and tonsillar crypt cultures, is of interest:

2/14.—Admitted to isolation ward. Dobell's gargle and spray every 4 hours; 10% silver nitrate swabbed on tonsils every other day. All cultures continued positive.

3/13.—Tonsillectomy.

3/14-3/28.—Dobell's gargle and spray every 4 hours.

3/28-5/ 1.—Argyrol, 10%, in nose and throat twice daily.

4/27-5/ 6-5/16.—Toxin-antitoxin mixture, 1 c.c. subcutaneously.

5/ 1-6/27.—Dobell's solution gargle and spray with alkaline antiseptic solution as gargle and spray every 4 hours.

6/21-7/31.—Dichloramine T., 2%, in oil swabbed in throat, and used as nasal spray 3 times daily.

7/31-8/15.—Still in isolation with positive nasal cultures.

The number of wound carriers was so small and the treatment so varied that we do not feel justified in forming any definite conclusion as to the best method of handling them. The following applications were used: Dakin's solution, irrigations at intervals of 2 hours, argyrol crystals, tincture of iodine, alcohol and saturated alcoholic solution of gentian violet. All of these antiseptics were used at different times on some of the cases. Daily cultures were made of all patients before the morning dressing. The following is an outline of the treatment of three of these wounds:

- R. D.: 5/15-5/20. Argyrol crystals on wound once daily.
 5/20-6/16. Tincture of iodine swabbed on wounds once daily.
 6/16-7/11. Dichloramine-T, oil solution once daily.
 7/11. Discharged as negative.
- J. R.: 3/20-3/30. Dakin's solution irrigations every 2 hours.
 3/30-4/30. Dichloramine-T, once or twice daily.
 4/30-5/15. Zinc oxide ointment once daily.
 5/15-6/22. Wound healed. Cultures positive until closed.
- H. N.: 4/ 9-4/19. Dakin's solution irrigations every 2 hours.
 4/20-4/30. Dichloramine-T twice daily.
 4/30-5/10. Dakin's solution irrigations every 2 hours.
 5/22-6/19. Tincture iodine once daily.
 6/19-6/28. Dakin's solution twice daily.
 6/28-7/18. Dichloramine-T twice daily.
 7/18-8/ 3. Gentian violet, saturated alcoholic solution, applied to granulating surfaces injected into granulating tissue and under the edges of the epithelium.
 8/ 3. Discharged from isolation with negative cultures.

Frequently when superficial cultures of these wounds became negative, the organisms could be readily obtained by curetting a small area and taking cultures from the tissues just below the surface. This fact was also noted by Simmons¹ in a series of thoracotomy wounds infected with diphtheria bacilli.

All cases of active wound diphtheria were treated with diphtheria antitoxin, given subcutaneously, intravenously or intramuscularly. The three following histories are illustrative:

R. W., aged 20, a white man, had measles, chickenpox, and whooping cough when small; pneumonia 1905. Tonsillectomy in 1908. No history of diphtheria. Gonorrhea in 1916.

¹ Jour. Infect. Dis., 1919, 25, p. 219.

On Aug. 8, 1918, Vesle River, France, shrapnel wounds both thighs and cheek bones (right). Left femoral artery severed by shrapnel. Bleeding checked and wounds bandaged at relief station; same night removed to evacuation hospital and left leg amputated at lower third of thigh. Sept. 14, to Base; Nov. 23 to U. S., and to Staten Island Hospital, where 3 inches of bone were removed from stump on Dec. 7; to Walter Reed General Hospital Dec. 27.

Condition on Admission: Sitting up in wheel chair, general condition good. Granulating area about 2 inches in diameter at site of incision in amputation wound. Stump swollen and red. Acutely inflamed.

Special senses normal. Skin and mucous membranes normal. Glandular system: enlargement of left inguinal glands; otherwise normal. Vascular system normal. Heart and lungs normal. Abdomen showed no abnormality. Liver and spleen not felt. Nervous system normal. Amputation at mid third of left thigh with inflamed granulating area about 2 inches in diameter.

Feb. 8: Unimproved. Hot packs, Feb. 12, Bact. count 30 per field.

Feb. 15: High bacterial count. Bichloride packs. Cultures: Staph. aureus.

Feb. 21: Stump culture showed diphtheria bacilli. Throat culture also positive for *B. diphtheriae*; admitted to isolation ward. Stump not tender. No membrane. Temperature normal. Diagnosis: Diphtheria carrier, in throat and wound. Schick test negative.

March 6: Released from isolation, after three negative cultures. No improvement. Dichloramine-T dressings.

April 1 to April 8: Dichloramine-T dressings.

April 8 to April 12: Dakin's q.2.h.

April 12: Stump became tender and painful to touch. Diphtheria bacilli present in cultures. Membrane began to form.

April 15: Admitted to isolation ward. Tough, dirty, white membrane over end of stump. Tightly adherent. End of femur protruding. Membrane extends $\frac{3}{4}$ inch up inner side of the thigh. Stump and surrounding skin tender and painful to touch. Has had hot boric compresses to wound since April 12. Temp. 100. General malaise.

April 17: No change. Given 10,000 units antitoxin. No reaction. Temp. 100.2.

April 18: Edges of membranes are loose and shriveling. Feels better generally.

April 19: Membrane gradually loosening; stump less tender.

April 20 and 21: No change. All membrane loose except small patch near femur.

April 22: Antitoxin 10,000 units subcutaneously.

April 23: Membrane hanging by small piece. Temperature 100.

April 24: All membrane gone. Temperature normal.

April 25: Dakin's solution q.2.h.

May 5: Healing rapidly.

May 15: Three negative cultures.

July 23: Cultures negative for diphtheria bacilli. Sent to operating room for shortening of femur.

R. T., aged 22, a white man, had measles, mumps and whooping cough in childhood, and pneumonia in March, 1918. No wounds. Denied venereal infections.

Sept. 14, 1918, on shipboard on way to France had influenza and pneumonia. Empyema developed in right lower side of chest and rib was resected in anterior axillary line. Remained in Brest, and tube was left in 3 weeks; back to U. S.,

Jan., 1919. Arrived at Walter Reed Hospital April 28, 1919. On admission had no cough or pain. Wound was draining slightly. Weight was normal, 144 lbs. General condition good. Over right lower side of chest percussion was dull, B & V sounds were distant. Otherwise physical examination was not remarkable.

April 29: Scar 3 inches in right anterior axillary line; almost healed.

May 22: Transferred to empyema service. No further note found on empyema service until May 23.

May 23: Large open wound, apparently gangrenous; no discharge of any consequence. Two large drainage tubes and hot packs.

May 25: Staphylococcus and *B. diphtheriae* in wound. Transferred to isolation ward.

May 25: Patient transferred to diphtheria ward. Temperature and pulse subnormal. At site of thoracotomy incision was a wide gaping deep wound, the surfaces of which were covered by a grayish membrane, which extended down to the deepest visible part of the wound. Immediately around the edges of the wound, the skin had sloughed off, leaving a shining, red, raw surface. This extended about an inch from the edge of the wound. Immediately beyond this area began a thin reddish gray membrane extending over the skin for about 4 or 5 inches around the wound. Just beyond the membrane there was a zone of inflamed skin which was sharply demarcated from the normal skin. Disagreeable odor noticed. Patient seemed in a very poor condition. He vomited all fluids and food, had not retained anything by mouth for 2 days. Complained of blurring of vision; 10,000 units of antitoxin were given intramuscularly on the 24th and the same amount on the 25th.

May 27: More of the membrane on the skin had disappeared, leaving the shining red surface for about 4 inches around the wound. The wound itself was unchanged. No spread of the process on the skin. Temp. was 97 by rectum, pulse averages from 50 to 60. General condition unchanged. Had serum rash over body; 10,000 units antitoxin were given intramuscularly; 500 cc physiologic salt solution subcutaneously. Vomited everything taken by mouth. Blood cultures taken on the 26th showed no growth.

May 28: Much of the membrane on the skin had shriveled and much had disappeared. Wound unchanged. Typical fetid odor persisted. Extensive rash over whole body. Continued to vomit. Morning temp. 96; pulse 50; blood pressure 90/60; 1000 cc of 10% dextrose and levulose solution intravenously. Had not voided since morning of 26th. Bladder was not distended. Condition poor. Evening temp. 95.5; pulse rate 40; blood culture showed no growth.

May 29: Membrane on skin practically all gone, and redness had also disappeared. Wound unchanged; 500 cc normal saline solution subcutaneously; 700 cc of 25% dextrose and levulose solution intravenously at the rate of 200 cc per hour. He had hiccoughs since early morning. About 3:00 p. m., vomited 500 cc of dark brownish material. Microscopic showed few red blood cells and much debris. Severe nose bleed. Blood CO₂ 37%. 6:20 p. m. began to spit up bright red blood, breathing became difficult, and he died at 6:30 p. m. Had not voided for 3 days; bladder not distended. Blood culture taken three days ago was still sterile.

Blood: May 28: Red blood cells, 4,448,000; white blood cells, 30,450; hemoglobin, 80%.

Differential Count: Polymorphonuclear neutrophils, 86%; small mononuclears, 8%; large mononuclears, 2%; transitionals, 4%.

Cultures of the throat for diphtheria bacilli were negative April 29, May 2, 4, and 28.

Cultures of the wound gave staphylococcus aureus and diphtheria bacilli May 25 and 27; cultures from all over the wound and surrounding skin May 28 gave diphtheria bacilli.

Necropsy: Thoracotomy wound with rib resection, right side; necrotic condition of edges; pleuritis organizing, right; petechial hemorrhages—skin, mesentery, omentum, stomach, intestines, epicardium, myocardium; pleuritis, chronic, left; lymphadenitis, bronchial, acute; carnification of lung right lower and middle; pneumonia, lobar, left lower; parenchymatous changes kidneys and liver.

Heart's blood: *B. diphtheria*; staphylococcus albus, streptococcus hemolyticus. Feces: *B. diphtheriae*. Lungs: *B. diphtheriae*; staphylococcus albus, streptococcus hemolyticus. Thoracotomy wound: *B. diphtheriae*.

M. L., aged 24, a white man, on Sept. 26, 1918, at the Verdun front, received a wound on the left buttock, from a high explosive shell; first aid in 6 hours; to Base hospital on 28th. Dressed daily until Nov. 15, when dislocated hip was reduced. Nov. 16, operated, incision and drainage. Dec. 1, incision and drainage of septic left knee. Dec. 20, incision and drainage of left thigh. Jan. 7, amputation of left thigh, at hip because of infection. Jan. 9, incision and drainage of abscess of left shoulder. Jan. 11, laryngeal diphtheria; received many injections of antitoxin. Feb. 15, pneumonia. Feb. 22, right side of chest aspirated. Sailed for U. S. March 8, and reached Walter Reed General Hospital April 11, 1919.

Condition on Admission: Unhealed disarticulation left thigh; undernourished; normal weight, 150 lbs.; present weight, 100 lbs. Teeth in bad condition. Gums: marked pyorrhea. Scar inner side left elbow. Entire right side of chest dull. Flat at base. Paresthesia fifth finger left hand. Large irregular scars left buttocks. Disarticulation of left thigh at hip. Three sinuses leading into hip. Marked general emaciation.

Progress: Wound treated and sinuses irrigated. Dakin's solution used.

May 5: Thoracentesis. No fluid. Roentgen ray showed increased density over lower right side of chest, less marked over upper part.

May 11: Thoracentesis; negative result.

June 9: General condition improved; less discharge from wound.

June 20: Thoracotomy. Incision and drainage C. D. dressing. Diagnostic puncture; nothing found. Culture taken this date showed gram-positive bacilli of the diphtheria group in thigh wound.

June 26: Admitted to isolation ward; *B. diphtheriae* positive.

June 27: Patient vomited all fluids and food given by mouth; pulse 120; temp. 97. The skin was dry and he seemed much in need of fluid. Wound showed an extensive thick diphtheritic membrane over most of the granulating surface. There was marked swelling and the wound was extremely painful. Over the skin surrounding the wound was a thin filmy grayish membrane, and just beyond the membrane was an area of erythema which was sharply demarcated and about 4 cm. beyond the edge of the membrane. Odor was fetid. Given 40,000 units of diphtheria antitoxin intravenously in the morning and 50,000 units intravenously in the afternoon; 500 c c normal salt solution intravenously; also proctoclysis.

June 28: Condition improved; pulse better volume; temp. 99 during night; 250 c c dextrose and levulose solution 25% intravenously at rate of 200 c c per hour. Hot boric dressing to wound. No change noted. Given 50,000 units antitoxin intravenously.

June 29: Continued to vomit. Large piece of membrane loosened and erythematous area of skin less. One liter 25% dextrose and levulose solution intravenously.

June 30: General condition about the same. Membrane about the same. Continued proctoclysis; 50,000 units of antitoxin in 600 c c normal salt solution intravenously.

July 1: Unable to retain anything by mouth. Edges of the membrane looser and a piece had sloughed off. Given 80,000 units of antitoxin in normal salt solution; 200 c c 25% dextrose and levulose solution intravenously. Pulse steadily weaker. Died 11:30 p. m. No urine voided during last 3 days of life. Bladder was not at any time distended.

Throat Cultures: April 11, *B. diph.* and *Strep. hemolyticus* absent. June 29, *B. diph.* absent.

Nasal Cultures: June 29, positive in both sides for *B. diph.*

Stump Cultures: June 20: Gram-positive bacillus of diphtheria group. June 25: *B. diphtheriae*. June 27: *B. diphtheriae* in skin and membrane on skin surrounding wound. June 29: All areas of wound positive for *B. diphtheriae*.

Necropsy: Abscess, post pleural, right; bronchopneumonia, right. Diphtheric infection, amputation wound; cloudy swelling of kidneys; peritonitis, generalized; amyloid degeneration, liver.

Bacteriology: Heart's blood: staphylococcus. Pericardium and pleural cavities sterile.

RESULTS AND DISCUSSION

The average stay per patient of the whole group of throat carriers admitted to the isolation wards from January to August was 22 days. These patients received only the routine treatment as described. The average period in isolation varied greatly from month to month; for instance, it was 28 days for those patients admitted in April; while for the month of May it was only 14 days. The treatment was the same for both months. Patients whose cultures were positive only once are not included in these averages. Some of these received the routine treatment while others were given none. In view of the great difference in the average length of stay in isolation from month to month and in view of the wide variation in stay of individual cases—6 days to 6 months—it seems that the form of treatment employed played little, if any, part in the removal of diphtheria bacilli from the individual throat. McCord and others² found that by standardizing their chlorcosane-dichloramine-T treatment, they reduced the average stay of convalescent carriers from 55 days in the first month of their report to 15 days in the last month; and the stay of contact carriers was reduced to 16 days for the last month of the report with an average of 23 days for 4 months. Our average for the whole time was 22 days; and for the last month it dropped to 14 days without any change in treatment. This may possibly have been influenced by the change in weather.

² J. Am. Med. Assn., 1918, 71, p. 275.

Our experience with tonsillectomy, though limited, seems worth mentioning. Six patients who had been given routine treatment in isolation, for about a month, and who gave positive tonsillar crypt cultures, were selected. These cultures were made after swabbing the tonsils thoroughly with a 2% solution of dichloramine-T. to eliminate surface contamination. The periods in isolation for these patients before and after tonsillectomy were:

TABLE 1
PERIOD OF ISOLATION BEFORE AND AFTER TONSILLECTOMY

Patient	Period of Isolation Before Tonsillectomy, Days	Period of Isolation After Tonsillectomy, Days
G. C.	114	15
D. A.	37	10
D. S.	32	11
R. T.	31	6
J. W.	29	15
W. W.	51	21
Average.....	54	11.3

These patients received the same gargle-spray treatment before and after operation.

The tonsils from these patients were placed in 10% formaldehyde for 10 minutes, after which they were opened, and cultures were made from their centers. Diphtheria bacilli were obtained from all. It is believed that tonsillectomy is the operation of choice in certain cases. A few carriers who had had their tonsils removed previously were carrying bacilli in tags of tonsillar tissue and cleared up after a second operation.

The average stay in isolation of 14 wound carriers was 29.8 days. Here again the form of local application used seemed to be of little importance. The periods of isolation varied from 8 to 90 days, and there was a wide variation in the period for patients with the same types of wounds and under the same type of treatment. For instance, one patient who carried diphtheria bacilli in an amputation stump wound which became negative after 9 days in isolation, had been treated with alcohol dressings for the first two days, after which he had received nothing but dry sterile dressings. Another patient with the same type of wound, who received alcohol dressings, remained positive for 40 days. Six patients with infected amputation wounds of the thigh, were treated with dichloramine-T. and exposure to sunlight, twice daily. Their average isolation period was 15 days, which was much shorter than

that for any other form of treatment, with the exception of the one case mentioned above. A number of cases were treated with dichloramine-T. alone without benefit. Careful cultural observations showed that the organisms were well below the surface of the wounds in practically all cases. In our experience the local applications mentioned above seemed to have little, if any, effect on the course of wound diphtheria carriers.

We had only 5 cases of active wound diphtheria and 2 of the patients died. In all of the cases, including the fatal ones, there was a marked decrease in the size of the membranes following the administration of diphtheria antitoxin. In one of the fatal cases, only 30,000 units of antitoxin were given, and the patient died of a diphtheria septicemia. Following the administration of 320,000 units of antitoxin in the other fatal case, about one-third of the membrane, which covered an area of 100 sq. cm. and extended over a large area of skin, loosened and came off. If the extent of the membrane is an indication of the degree of toxicity, extremely large doses of antitoxin are indicated in these cases. The immediate cause of death was probably peritonitis and septicemia, due to a staphylococcus infection, and not the diphtheria infection which was steadily improving. In the three patients that recovered, the membranes were not so extensive and disappeared promptly after the administration of from 20,000 to 30,000 units of antitoxin. In both of the fatal cases, the patients became extremely desiccated, and this factor was very troublesome. It appeared in both cases about 5 days before death, shortly after vomiting began. Intravenous administration of fluids was necessary as the emaciated condition of the patients did not permit a sufficient amount subcutaneously, and they were unable to retain any appreciable amount of fluids given by rectum. This desiccation, perhaps, could be prevented by giving a sufficiently large amount of antitoxin early in the course of the disease, but once present care should be taken to keep up a liberal fluid intake daily.

BACTERIOLOGIC

All primary cultures were made on Loeffler's serum and incubated at 37.5 C. for from 18 to 20 hours. Positive diagnoses were made only when Wesbrook's types A, B, C or D were found in stained preparations. Pure cultures were obtained by replating on horse whole blood agar, or serum agar. The diphtheria bacilli from 177 primary cultures all retained Gram's stain and showed polar granules when stained with methylene blue and by Neisser's method. No acid-fast organisms or granules were found.

VIRULENCE

Fifty-two strains, including 27 from contact throat carriers, 13 from convalescent throat carriers, 7 from wound carriers and 5 from wound cases, were tested to determine their virulence for guinea-pigs. Animals weighing 300 gm. were injected subcutaneously with 1.5 cc of a 72-hour broth culture, and in each instance control animals, inoculated intraperitoneally 24 hours previously with 100 units of diphtheria antitoxin, were given a similar dose. The percentage of virulent strains obtained from contact throat carriers and wound carriers were 48.1 and 42.8, respectively; while those from wound cases were 80% and from convalescent throat carriers 84.6%. Organisms of low virulence which killed guinea-pigs after 2 weeks, with typical local reactions, were not included in these percentages.

TABLE 2
VIRULENCE FOR GUINEA-PIGS

Source of Culture	Number Tested	Very Virulent		Low Virulence		Avirulent	
		Number	Per Cent.	Number	Per Cent.	Number	Per Cent.
Contact throat carriers.....	27	13	48.1	1	3.7	13	48.1
Wound carriers.....	7	3	42.8	1	14.2	3	42.8
Convalescent throat carriers.....	13	11	84.6	1	7.6	1	7.6
Wound cases.....	5	4	80.0	1	20.0	0	0.0
Total.....	52	31	59.6	4	7.6	17	32.6

CULTURAL CHARACTERISTICS

All cultures on Loeffler's serum were moist, confluent and grayish white. A few strains developed a yellowish tint after several days' incubation; none were proteolytic. The growth on whole blood-agar plates was moist, confluent, opaque and dark gray in color. A slight degree of hemolysis occurred with a few strains. The reactions with litmus milk were not striking; a small amount of acid was produced by three strains—no coagulation or clearing occurred. Pellicle formation with sedimentation was observed in all dextrose-broth cultures.

FERMENTATION OF CARBOHYDRATES

Tubes containing 1% each of C. P. glucose, saccharose, maltose, dextrin, inulin, mannite, lactose, xylose and arabinose, in 10 c.c. of sugar free infusion broth, were inoculated with standard amounts of pure cultures from each of 32 strains. The phenol red-china blue indi-

cator of Morshima³ was used, and the reaction of all mediums was made P_H. 7. Cultures were incubated at 37.5 C., and 4 readings were made at intervals of 2 days. Uninoculated tubes of each kind of carbohydrate broth were incubated as controls; and final readings were made by comparing the colors of the various cultures with a set of standard solutions. Twenty-three of the strains were virulent and 10 were avirulent; 17 were obtained from contact throat carriers, 8 from convalescent throat carriers, 2 from wound carriers and 6 from wound cases.

TABLE 3
FERMENTATION OF CARBOHYDRATES

Source of Cultures	Total	Virulence of Guinea-Pig	Glucose	Saccharose	Maltose	Dextrin	Inulin	Mannite	Lactose	Arabinose	Xylose
Contact throat carrier.....	6	Very virulent	AC	ALK	AC	AC	ALK	ALK	*	NC	NC
Convalescent throat carrier...	5		AC	ALK	AC	AC	ALK	ALK		NC	NC
Wound case.....	3		AC	ALK	AC	AC	ALK	ALK		NC	NC
Wound carrier.....	1		AC	ALK	AC	AC	ALK	ALK		NC	NC
	— 15										
Contact throat carrier.....	4	Virulent	AC	ALK	AC	AC	ALK	ALK		NC	NC
Convalescent throat carrier...	3		AC	ALK	AC	AC	ALK	ALK		NC	NC
	— 7										
Contact throat carrier.....	3	Avirulent	AC	ALK	AC	AC	ALK	ALK		NC	NC
Nose of fatal wound case.....	1		AC	ALK	AC	AC	ALK	ALK		NC	NC
Wound carrier.....	1		AC	ALK	AC	AC	ALK	ALK		NC	NC
	— 5										
Contact throat carrier.....	1	Avirulent	AC	NC	AC	NC	ALK	ALK	NC	NC	NC
	— 1										
Wound case.....	1	Virulent	AC	AC	ALK	ALK	ALK	ALK	ALK	NC	NC
	— 1										
Contact throat carrier.....	3	Avirulent	AC	AC	ALK	ALK	ALK	ALK	ALK	NC	NC
Wound case.....	1		AC	AC	ALK	ALK	ALK	ALK	ALK	NC	NC
	— 4										

AC = acid, ALK = alkaline, and NC = no change.

* Reactions of lactose broth cultures were variable in first group; no change in 21, slightly acid in 4 and alkali in 2.

All of the 33 strains produced acid (P_H. 5.4-5.6) in glucose, and alkali (P_H. 7.3-7.6) in mannite and inulin; while no change occurred in the reaction of xylose or arabinose broth cultures. Saccharose cultures were made more alkaline (P_H. 7.4-7.6) by 27 strains; acid was produced by one avirulent and one virulent wound case strain, and by 3 avirulent contact throat carrier strains; and no change in reaction occurred with one avirulent contact throat carrier strain. Maltose was fermented (P_H. 5.7-6) by all, except 1 avirulent and 1 virulent wound case, and 3 avirulent contact throat carrier strains. These 5 cultures showed an increase in alkali (P_H. 7.3-7.6).

³ J. Infect. Dis., 1920, 26, p. 43.

Acid (P_H 6.4-6.7) was produced in dextrin broth by 27 cultures. There was no change in the reaction of one avirulent contact throat carrier culture; and alkali (P_H 7.2-7.4) was produced in 3 avirulent contact throat carriers; one avirulent and one virulent wound case culture. Twenty-two strains produced no change in the reaction of lactose broth; 6 produced acid (P_H 6.5-6.6) and 7 produced alkali (P_H 7.4). As usual, the fermentation reactions gave no indication of the virulence of the organisms tested.

None of the strains produced indol in Dunham's peptone.

BLOOD CULTURES, ETC.

Blood cultures were made of 43 contact throat carriers, 9 convalescent throat carriers, 3 wound carriers and 2 wound cases; all were sterile with one exception. An atypical granular diphtheria-like bacillus was obtained in one flask of a blood culture from M., who was a contact throat carrier. This organism, however, differed culturally from diphtheriae and produced a fatal septicemia in guinea-pigs in spite of diphtheria antitoxin immunization. As a growth occurred in only one flask, this might have been a skin contamination. Urine cultures made from centrifugalized specimens of 26 carriers were all negative. Unclassified granular bacilli were obtained in 11 of them. No diphtheria bacilli were found in feces cultures of 21 carriers. A small amount of feces was broken up in glycerol and allowed to stand at room temperature for 48 hours, after which the precipitate was streaked on blood-agar plates and incubated.

The blood of 25 carriers was grouped for isohemo-agglutinins according to Jansky with the following results: group 1, 1 (4%); group 2, 6 (24%); group 3, (12%) and group 4, 15 or 60%. (Moss classification.)

Nasal cultures were positive on 30 of 44 contact throat carriers, 8 out of 9 active throat cases and 4 of 5 infected wound cases. Four of 5 wound cases had positive tonsillar surface cultures.

Tonsillar crypt cultures made from 44 contact throat carriers were positive in 24 instances, 7 of 9 active throat cases and 2 of 5 infected wound cases were positive.

Eight of 44 contact throat carriers and 1 of 5 wound cases had had their tonsils removed at some previous time. All of these were carrying bacilli in small tags of tonsillar tissue.

Complete blood counts and urine examinations made of all carriers failed to show any characteristic variation from the normal.

SUMMARY

Between October, 1918, and August, 1919, 75 cases of diphtheria and 102 carriers of diphtheria bacilli were treated at the Walter Reed General Hospital.

New cases continued to develop in the hospital each week, in spite of the isolation of all positive contacts until the summer months when the occurrence of diphtheria is generally lowest.

The successful treatment of carriers depended not so much on the kind of antiseptic used but on the ability to reach the organism with the antiseptic, or when possible on complete removal of the infected foci.

The danger of active wound-infection should be recognized and early treatment with large doses of diphtheria antitoxin should be given.

Forty-eight and 42%, respectively, of the strains from contact throat carriers and wound carriers were very virulent, while 84.6% of those from convalescent throat carriers and 80% of those from wound cases were very virulent.

Neither morphology, fermentation reactions nor any other cultural characteristic gave any indication of the degree of virulence of the organisms studied.

No diphtheria bacilli were found in cultures of the blood, urine or feces of cases or carriers, except from one fatal wound case post-mortem.

NOTES ON SOME BACTERIAL PARASITES OF THE HUMAN MUCOUS MEMBRANES

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The following observations are offered in the hope that they may be of service to others who may take up the study of the extensive and imperfectly described bacterial flora of the human mucous membranes.

1. BACTERIUM MELANINOGENICUM (N.SP.)

The occurrence of black colonies on anaerobic blood-agar cultures from carious teeth, etc., has been noted by several investigators. No one, so far as we are aware, has isolated and described the organism. We have isolated it from the throat, tonsils, infected surgical wound of the abdomen, from urine collected as aseptically as possible from a suspected focal infection of the kidney, and from the feces of a case of chronic dysentery superimposed on an original amebic infection.

Isolation and Biologic Characters.—The bacterium grows readily at 37 C. on + 1 human blood-agar slants made anaerobic by the pyrogallie acid method. In primary cultures, the colonies become visible within one to two weeks. Pure subcultures yield a confluent, black, dry layer of growth (fig. 1), within which lie the somewhat polymorphous rods, embedded in the masses of pigment. As the growth increases the hemoglobin throughout the agar slant disappears, until finally the medium appears like a slant of plain agar. The growth of the bacterium on infusion agar is greatly facilitated by the presence of hemoglobin, and pigment production is absolutely dependent on it.

In sodium phosphate broth (+ 1) the organism produces acid from dextrose, levulose, lactose, saccharose, maltose and mannite. Galactose is not attacked. On potato no growth occurs, nor does growth occur on agar or broth made from Liebig's beef extract, with or without dextrose or maltose. In gelatin containing pleuritic fluid there is a dense flocculent growth at 37 C., but no liquefaction. On Loeffler's blood serum a fairly luxuriant, white, moist layer of growth develops. Litmus milk is slowly acidified, but not coagulated.

The bacterium occurs as somewhat polymorphous rods, in size about $0.8\ \mu \times 1\ \mu$ — $3\ \mu$ (fig. 2). They lose the stain in Gram's

method and are nonacid-fast. Motility is absent in both sealed and unsealed preparations.

The rods themselves are unpigmented. The pigment occurs as extracellular, amorphous masses, and apparently is a melanin. The pigment is insoluble in alcohol, ether, chloroform, acetone, carbon-disulphide and carbon-tetra-chloride. It slowly dissolves in sodium hydroxide solution.

2. *B. DUPLEX-NONLIQUEFACIENS* IN BRONCHIAL SPUTUM

This bacterium has been encountered not infrequently in the mucopurulent sputum of acute and chronic bronchitis. It seemed to be particularly prevalent in Cincinnati in 1915. Often it was present in the bronchial sputum in quite as large numbers as the members of the pneumococcus group (fig. 3). According to Scarlett,¹ this species sometimes occurring in ulcer of the cornea can easily be distinguished from the diplobacillus of Morax-Axenfeld and that of Petit by means of specific agglutinating serums.

Our cultures corresponded with *B. duplex* in their cultural characters, except that they failed to liquify blood serum slants. Particularly luxuriant growth was obtained on slants of ascites agar grown at 37 C. under partial tension with the hay bacillus. In such cultures, the diplobacillus arrangement was retained (fig. 4).

A whole ascites-agar slant culture was injected into the pleural cavity of a rabbit. When the rabbit was killed and examined a month later it appeared normal.

3. *M. MINUTISSIMUS* (N.SP.)

This organism, apparently an obligate anaerobe, was isolated from the mixed flora in the aphthous ulcers of the gingival and buccal mucosa of a case of postpoliomyelitic paralysis. It appears as spherical, nonmotile cocci and diplococci less than 0.5 μ in diameter (fig. 5, in which they are photographed along with *S. aureus*). The coccus stains readily with the ordinary aniline dyes but loses the stain in Gram's method.

When grown anaerobically by the pyrogallie acid method on sodium phosphate nutrient agar containing pleuritic fluid, discrete colonies are formed. They are opaque, white and attain a diameter of 1 mm. When grown anaerobically by the pyrogallie acid method on sugar-free

¹ Ann d'Oculistique, 1916, 153, 485.

phosphate agar (+ 0.8 to phenolphthalein) containing 1% of dextrose, levulose, galactose, lactose, saccharose, maltose, mannite and pleuritic fluid, the growth was more luxuriant than on plain pleuritic fluid agar, and bubbles of gas appeared in the water of syneresis. If litmus was present in the above sugar mediums, after 4 days' growth at 37 C., the litmus was reduced. On opening the tubes, they turned a deep blue color. In sugar-free sodium phosphate broth a large bubble of gas was produced which was not absorbed by sodium hydroxide and was noninflammable. An attempt to adapt the organism to aerobic growth failed.

A rabbit was inoculated intravenously and a guinea-pig and white mouse intraperitoneally, each with a whole slant of the culture. None of the animals were infected.

4. *M. RENIFORMIS* (DIPLOCOCCUS *RENIFORMIS*, COTTET, 1900)

This coccus was isolated in ascites-agar cultures, made anaerobic by the pyrogallic acid method, from the vaginal pus of a case of vulvovaginitis in a child. Numerous gonococci were present in the pus, as revealed by direct smears and cultures incubated at partial tension.

M. reniformis tends to occur as bean-shaped diplococci slightly smaller than the gonococcus. In cultures, it retains a fairly uniform size, without the production of the swollen involution forms which so soon appear in cultures of the gonococcus. It loses the stain in Gram's method, but is somewhat more slowly decolorized than the gonococcus.

The growth of this strictly adapted anaerobe ceased at 6 mm. from the surface of a stab culture in ascitic agar. It died on exposure to the air. Growth failed to occur on Loeffler's blood serum, plain nutrient agar or broth, milk and potato. During 8 days' incubation at 37 C. it did not ferment dextrose, levulose, galactose, saccharose, lactose, maltose and mannite. After 8 days' incubation at 37 C. in indol-free broth containing sodium phosphate and ascitic fluid, a marked reaction for indol was obtained with H_2SO_4 and KNO_3 .

We presume that our culture is probably identical with that of Cottet,² although he merely records the morphology, reaction to Gram's stain, strict anaerobiosis and the character of its growth on agar and gelatin. He found that the organism produced a localized abscess when inoculated into the skin of a guinea-pig and smears made from the pus resemble those from a case of gonorrhea rather closely. Cottet called attention to the danger of mistaking it for the gonococcus in smears and

² Compt. rend. Soc. de biol., 1900, 52, p. 421.

to its probable pathogenicity. It was encountered by him in four cases of abscess of the urinary tract, in the pus from a case of pyonephrosis, and in almost pure culture in the urine of a case of cystitis. He states that Veillon and J. Halle, according to a verbal communication, found it in mixed culture in a case of gangrenous inflammation of the vestibule of the vulva of a little girl.

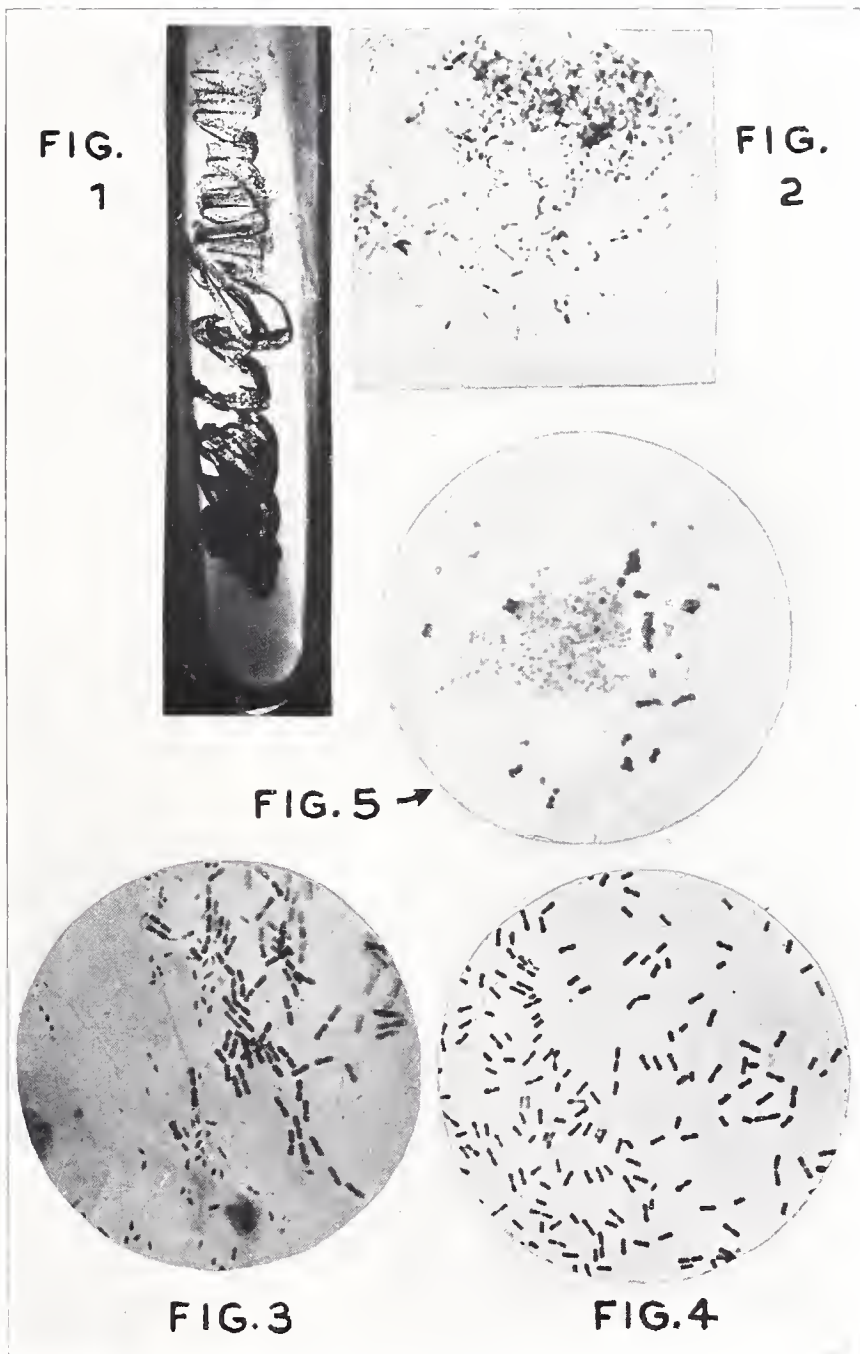


Fig. 1.—*Bacterium melaninogenicum* (N. Sp.) anaerobic blood-agar slant culture.
 Fig. 2.—*Bacterium melaninogenicum* from an anaerobic blood-agar culture showing the rods lying among pigment granules; \times about 1,000.
 Fig. 3.—*B. duplex-nonliquefaciens*. Smear from bronchial sputum stained with gram-safranin; \times about 1,000.
 Fig. 4.—*B. duplex-nonliquefaciens* from partial tension ascites-agar culture; \times about 1,000.
 Fig. 5.—*M. minutissimus* from anaerobic pleuritic fluid agar culture mixed with *Staphylococcus aureus* and stained by gram-safranin; \times about 1,000.

A STUDY OF AN ORGANISM FROM NEPHRITIS IN SHEEP

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In 1918 Fitch, Boyd and Billings¹ reported on the isolation of an organism from ovine nephritis. Since then we have isolated a similar organism from two other cases and have made a study of its more important characteristics.

Ovine nephritis is not commonly reported in the literature. According to Leblanc acute nephritis is almost unknown among sheep. Baker² does not mention it, nor does Oppermann.³

The three cases from which we have isolated the organism here described occurred in rams. The symptoms in each case were obscure, and a diagnosis of the real cause of the trouble was not made until necropsy.

The necropsy findings in the first case were: "The kidneys showed active inflammation; the capsule was adherent and when removed particles of pus were discharged from small abscesses located in the cortical region. In the pelvis there were several cavities (1-2 cm.) which were filled with pus. The ureters were enlarged, hyperemic, and edematous and contained some pus. The musculature of the bladder was not involved, but the mucous surface was inflamed and studded with hemorrhages of punctiform variety. Microscopic examination of properly stained sections showed the typical changes of a purulent nephritis."

The cultural findings as reported in this first article were as follows: "Cultures made on nutrient agar from the kidneys and incubated at 37 C. showed sparse growth. After three days' incubation a few small grayish white colonies appeared on each of the cultures. Microscopic examination showed a short (1-2 μ) rod-shaped and non-motile organism, with rounded ends, existing usually singly, sometimes in pairs and short chains. It stains well with carbol fuchsin and methylene blue. It is gram-negative. Grows quite vigorously in infusion bouillon with the production of considerably rather viscid sediment. This growth appears at first in the top layers of the bouillon and then gradually settles. It has no action on milk. On agar the organism grows very sparingly with the production of small grayish white colonies. The growth in

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¹ Cornell Veterinarian, 1918, 8, p. 241.

² Diseases of Sheep, 1920.

³ Lehrbuch der Krankheiten des Schafes, 1919.

bouillon containing glucose, lactose and sacchrose is essentially the same as described in plain bouillon. No gas is produced in any of these sugars nor is the reaction changed to litmus."

This organism died out, and we did not have another case until January, 1920. This sheep was sick 48 hours. Digestive disturbances were suspected, but on necropsy an acute nephritis and an inflammation of the mucous membranes of the bladder and ureter were found. Cultures were made on slant agar from the kidneys and bladder. From the kidneys an organism grew in pure culture. From the mucous surface of the bladder the cultures were mixed, some containing an organism of the *B. coli* type and many colonies of the same organism found in the kidneys. This organism is designated as strain B.

In June, 1920, an Oxford ram was found dead in the pasture. The animal had been noted as not doing well for 2 weeks previously and stomach worms were suspected as the cause. Necropsy showed that the kidneys were enlarged about twice. The ureters were enlarged and the mucous membrane highly congested. The mucous surface of the wall of the bladder was congested and in places hemorrhagic. The kidneys showed ecchymotic areas (0.5 to 1 cm.) on the surface beneath the capsule. The capsule was slightly adherent. Cultures from the cortex of the kidney on plane agar gave very slight growth. Cultures prepared from the medulla and pelvis on incubation showed many colonies of *B. coli-communis*. Cultures prepared on serum agar from the cortex showed many small white colonies which on further study were shown to be closely allied to the strain mentioned previously, and hereafter known as strain F.

Morphology.—The organism is a short 1-2 μ rod with rounded ends. Occasional coccoid forms are seen, especially in old agar slant cultures. Sometimes long involution types are noted from agar cultures. It is nonmotile, and no spores or granules are visible. It stains readily with the aniline dyes. It does not, however, take Gram stain.

Cultural Characteristics.—In meat infusion broth, especially in early generations, growth is sparing, becoming visible in 48 hours. In later generations growth is moderate in 24 hours. Cloudiness is uniform with a developing semiviscid white sediment which also adheres to the sides of the tube and forms a viscid terminal ring. In broth containing certain of the carbohydrates, a thin surface pellicle is formed. In cultures incubated for 5 days, the sediment becomes quite heavy. Indol is not produced in Dunham's peptone and growth is very sparing.

On beef infusion agar, especially when first isolated, growth is very scant. On agar containing 10 per cent. horse serum growth is much more vigorous. The colonies are discrete, grayish white, translucent and show a bluish irides-

cence. After 4 generations of artificial cultivation, the growth becomes more confluent and filiform. The growth shown by the F strain after long continued cultivation tends to become heavier than that of the B strain.

On gelatin the strains will not grow at 20 C. Growth at 37 C. was slight and no liquefaction was produced. Growth in incubated gelatin was restricted to a few colonies on the surface.

Small white colonies are produced on Loeffler's serum; no liquefaction takes place.

No change is noted in milk after 7 days' incubation. Litmus milk likewise shows no change in reaction.

Strains B and F were streaked on defibrinated blood agar. One c c of blood was used for 10 c c of agar. No hemolysis was noted after 48 hours' incubation at 37 C.

Fermentation Studies.—A careful study was made of the change in the hydrogen-ion concentration in sugar-free beef infusion broth containing various carbohydrates. The method followed was the one described by Medalia⁴ in determining the P_H values of culture mediums. We did not follow the method described by him to determine the effect of organisms on indicators. The indicator studies were checked from time to time by the potentiometer. The various carbohydrate mediums were sterilized by filtration as described by Fitch and Billings.⁵ The details of this work are contained in table 1.

TABLE 1
AVERAGE P_H VALUES OF EACH STRAIN IN DUPLICATE IN VARIOUS CARBOHYDRATES *

Strain	Strain B		Strain F		Uninoculated Controls
Arabinose.....	6.8	7.0	6.2	6.3	6.8
Xylose.....	6.6	6.7	6.4	6.3	6.8
Rhamnose.....	7.2	7.3	7.2	7.2	7.2
Dextrose.....	7.5	7.4	6.5	6.5	7.0
Levulose.....	7.3	7.3	6.0	6.0	7.0
Galactose.....	7.4	7.3	6.3	6.3	7.1
Sucrose.....	7.2	7.4	7.1	7.1	7.0
Maltose.....	6.9	7.1	6.1	6.2	7.0
Lactose.....	7.2	7.2	7.0	7.0	7.0
Raffinose.....	7.5	7.6	7.3	7.3	7.1
Inulin.....	7.6	7.6	7.1	7.1	7.0
Salicin.....	7.5	7.5	7.2	7.3	7.0
Mannitol.....	7.1	7.2	6.7	6.7	7.0
Dulcitol.....	7.4	7.5	7.3	7.3	7.2

* The original tables giving the P_H value for each titration can be secured from the authors.

The carbohydrates were inoculated in duplicate with strains B and F, then incubated at 37 C. and the reactions determined on the 1st, 3rd, 5th, 10th, 20th and 30th day of incubation. Readings were made in terms of H-ion concentration. In order that space might be conserved we have condensed the original tables into one. The P_H values appearing in this table were compiled by adding together the 6 P_H values obtained on each day (1st, 3rd, 5th, etc.) and recorded for the strain in the various carbohydrates. Then an average titration figure was obtained from the sum of the 6.

⁴ J. Bacteriology, 1920, 5, p. 441.

⁵ Ibid., p. 469.

Summarizing the results, we find that in arabinose and xylose strain B practically does not change the P_H value with a possible slight production of acid in xylose, while strain F produces a slightly acid reaction in both. Neither strain changes the reaction of rhamnose. In the hexoses the F strain produces acid while the B strain produces alkalinity. Sucrose is unchanged by both strains until the 30th day when a decided alkalinity is produced by both strains, so as to raise the average P_H value slightly above the controls. In maltose, strain B produces no change, while F produces a marked acid reaction. In lactose both strains remain practically unaltered, strain B having a slightly higher P_H value.

Raffinose of the trisaccharid group is rendered alkaline by both strains. Inulin of the polysaccharids is rendered alkaline and is more pronounced by the B strain.

Salicin of the glucoside group is rendered slightly alkaline more pronounced by the B strain.

Of the polyhydric alcohols, mannitol is rendered alkaline by the B strain while the F strain produces a slight acidity. In dulcitol a slight alkalinity is noticed more pronounced by the B strain.

Gas is not produced in any of the carbohydrate broths. The inverted vial method was used.

Several interesting observations were made in this study. We found that certain of the carbohydrate mediums in tubes with paraffined plugs had a different P_H value than in similar tubes with unparaffined plugs. This difference was noted in both inoculated and uninoculated tubes after 10, 20 and 30 day incubations. This difference apparently was not due alone to the effect of the organism but was in part caused by the continued incubation. A careful study of this feature is being planned.

Inoculation and Immunologic Experiments. It was stated in the first report on this organism that: "Subcutaneous inoculations into rabbits and guinea-pigs are yet (5 weeks) attended with no results." We have repeatedly given intravenous, intraperitoneal and subcutaneous inoculations of various quantites of fresh cultures of this organism into rabbits and guinea-pigs without apparent bad effect. The inoculated animals have remained well for two months, after which they have been killed and careful necropsy examinations have been made. No lesions have been found.

We injected one ram (1) intravenously with 2 c.c. of the F strain. This material was secured by washing off the growth from two 24-hour serum-agar cultures with 5 c.c. salt solution. These cultures were the 4th generation of this strain after original isolation. At the same date we injected a second ram (2) intraperitoneally with 4 c.c. of a broth suspension from 2 slant agar cultures of the B strain. These injections were made at 10 a. m. The temperature of sheep 1 when injected was 102.5, at 12:15 p. m., 105, at 6 p. m. 105.8. Animal in visible distress. Dyspnea, bloody diarrhea, and bloody mucus from rectum. Died early the next morning. A careful necropsy examination was performed, the principal changes being congestion of the various internal organs, sero-sanguineous fluid in the thoracic cavity, and marked congestion and hemorrhages of the mucosa of the intestinal tract. Clots of blood were found in the colon. The kidneys were markedly congested. Cultures from the lungs showed the same organism as strain F. Cultures from the kidneys remained sterile. Portions of kidneys taken for microscopic examination showed parenchymatous degeneration, congestion and hemorrhage.

The temperature of ram 2 continued normal, and no evidence of disease was noted. This animal was killed some weeks later. No macroscopic evidence of disease was found. Sections of the kidneys on microscopic examination showed no evidence of change.

Ram 3 was injected subcutaneously with 2 c c of salt solution suspension of F strain prepared in the same manner as the foregoing. On the 5th, 6th and 7th days following injection, a rise of temperature to 104.6 was noted. This, however, fell to normal on the 8th day. This animal became emaciated and weak and died after 30 days, and the necropsy examination showed pleuritic adhesions, serosanguineous fluid in the thoracic cavity, pneumonia and consolidation of the anterior lobes of the lungs. One specimen of *Dictyocaulus filaria* was found in a consolidated portion. A few *Hemonchus contortus* were present in the abomasum. The kidneys were apparently normal. Cultures on horse serum agar from the various organs, incubated at 37 C. did not yield strain F from any of the organs. Microscopic sections of the kidneys showed parenchymatous degeneration.

Ram 4 was injected intravenously with 0.5 c c of suspension of F strain. On the afternoon of the same day the temperature rose to 105.2 F. The following day it was back to normal and remained so until the 5th, 6th and 7th days, when it rose to 104.2. On the 8th day the temperature again became normal and remained so. No apparent evidence of disease was noted. This animal was killed 10 weeks later and all organs appeared normal, microscopic sections of the kidneys showing no pathologic changes. Cultures from the various organs remained sterile.

Ram 5 was injected intravenously with 2 c c suspension of F strain. The temperature on following day rose to 106 F. The animal was dull. The temperature returned to normal on the next day. On 7th, 8th and 9th days, it rose again to 104.3. During this period the animal was off feed. Normal temperatures were observed thereafter. This animal was killed about 10 weeks later and appeared macroscopically normal. Cultures on serum agar from the various organs remained sterile. Microscopic sections of the kidneys showed congestion and evidence of chronic nephritis with leukocytic infiltration, especially in the cortex.

Ram 6 was injected intravenously with 2 c c suspension of B strain. A rise of temperature to 104.3 F. was noted on the 5th day. This animal was off feed for 9 days. No further evidence of disease was noted, and the animal was killed 10 week later. A subcutaneous abscess 2.5 c. m. in diameter was found at point of injection. No other macroscopic lesions were noted. Cultures made on serum agar from the kidneys, testicles and subcutaneous abscess remained sterile. Those from the lungs, liver and spleen showed colon type organisms. Microscopic examination of pus from the subcutaneous abscess showed gram-negative short rods. Sections of the kidneys showed microscopically the same changes as noted in ram 5.

An effort was made to inject into the urethra of ewe lamb 7 a suspension of the F strain. We were unable to make the injection, directly into the urethral orifice and the suspension was placed in the vagina at the urethral opening. The temperature of this animal remained normal for 5 days following the inoculation. About 6 weeks later the animal did not eat well, had lost flesh, and was growing very weak. Fourteen days later the animal could not stand, and was killed. Both kidneys showed marked evidence of chronic nephritis. The capsules were adherent and the cortex gray in color. A very few *Hemonchus contortus* were found in the abomasum. Other organs were apparently normal. Cultures

were made on serum agar from the various organs, and on incubation remained sterile. Microscopic sections of the kidneys showed marked parenchymatous degeneration, especially in the cortex. The glomeruli were also involved.

Blood was drawn from sheep 5, 6 and 7. The serums from these animals agglutinated strains F up to and including a 1:1000 dilution.

Strain B was not agglutinated at all. Standard technic was used.

In interpreting these results, it must be kept in mind that sheep 5 and 7 were inoculated with strain F, while sheep 6 was inoculated with strain B. It is difficult to understand the reaction obtained.

Rabbits were immunized to strains B and F, respectively. The injections were given as follows: 1 cc salt solution suspension, intravenously Sept. 25; 3 cc salt suspension, subcutaneously Oct. 2; 5 cc salt suspension, subcutaneously Oct. 12. Animals bled Oct. 20.

Serum from rabbit immunized with strain F agglutinated strain F completely up to 1:200 and slightly in the 1:500 and 1:1000 dilution. Strain B was agglutinated in the 1:20 dilution and slightly in the 1:50.

Serum from rabbit immunized to strain B agglutinated strain B up to 1:200, while strain F was negative throughout. These same rabbits were reinjected as follows: 3 cc suspension intravenously Oct. 28; 5 cc suspension intravenously Nov. 8; 5 cc suspension intravenously Nov. 20.

The rabbits were bled Dec. 27. The serum reactions were: Serum from rabbit immunized to strain F, agglutinated strain F up to 1:1000, while with strain B it gave a partial reaction in all tubes up to 1:200. Serum from rabbit immunized to strain B agglutinated both strains up to and including 1:1000.

DISCUSSION

A consideration of the foregoing data shows many things which require further study. The 2 strains from nephritis in sheep have certain cultural differences as shown in their effects on the various carbohydrates. However, it is hardly justified to separate them into different species on the basis of such minor variations. Another factor which may influence the variations, is the effect which incubation has on carbohydrate mediums as denoted by changed hydrogen-ion concentration. With phenolphthalein as an indicator of change in the reaction of the various carbohydrate mediums inoculated with the organisms, the variations in the two strains as denoted by the P_H values were not observed.

The inoculation experiments indicate that it is not easy to reproduce nephritis in sheep and recover the organism. The experience with sheep 7 indicates that the infection may be an ascending one through the bladder and ureters. Further work on this point is under way.

The immunologic relationship of the 2 strains are peculiar. However, they are no greater than those reported in the same species

by Meyer and Shaw^{5a} in their immunologic studies with *B. abortus* and *B. melitensis*.

It is interesting to compare the biologic and pathogenic characters of the organism here described with those of an organism producing nephritis of horses and joint ill of foals. This organism was originally described by Theiler and Meyer⁶ in South Africa in 1908. Recently this same organism has been studied by M'Fadyean and Edwards⁷ in England and Magnusson in Sweden.⁸ In many respects, morphologically there is a strong resemblance between this organism known as *Bacillus nephritidis* (Meyer), or *Bacterium viscosum equi* (Magnusson) and those isolated by us from sheep. Culturally there are variations, such as marked viscosity of growth by the equine organism, which is not shown to such an extent by the sheep strains. Pathogenically the equine organism seems to be specific for horses and the one here described, for sheep. Nephritis is produced by both. We have endeavored to obtain from M'Fadyean a strain of *Bacillus nephritidis* but he states that it is short lived and his strains are just now dead. We anticipate, however, a careful comparison of these two organisms. A study of the pathologic changes in nephritis of sheep is under way, and will be presented in another paper.

CONCLUSIONS

An organism for which we propose the name *Bact. nephritidis* var. *ovis* has been isolated from three cases of ovine nephritis.

Two strains of this organism have slightly different PH values in various carbohydrate mediums.

Further experiments are necessary before the channel of infection of this organism can be told with certainty.

This organism is nonpathogenic for guinea pigs and rabbits.

Apparently a somewhat close relationship exists between the organism here described and *Bacillus nephritidis-equi*, Meyer.

The serologic investigations do not yet permit positive conclusions.

^{5a} J. Infect. Dis., 1920, 27, p. 173.

⁶ Transvaal Dept. Agriculture, Report of Government Veterinary Bacteriologist, 1908-9.

⁷ Jour. Comp. Path. and Therap., 1919, 32, p. 42.

⁸ Ibid., p. 143.

A STUDY OF THE GASEOUS REQUIREMENTS FOR THE GROWTH OF VARIOUS BACTERIA

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It is generally believed that most bacteria require oxygen for their growth and that the oxygen is obtained directly from the atmosphere, or indirectly through enzyme action from carbohydrates, proteins or other reducible bodies.

That the relation of the growth of bacteria to their gaseous environment might not be quite so simple was suggested by work on the so-called partial tension strains of bacteria; and especially by the observation of Wherry and Ervin¹ that the removal of the CO₂ given off by the bacteria prevented the growth of a recently isolated strain of the tubercle bacillus.

EXPERIMENTS

In a series of experiments during 1919, it was noted that many strains of bacteria—a saprophytic tubercle bacillus, the hay bacillus, staphylococcus aureus, and the gonococcus—would grow very well under anaerobic conditions as provided by displacement with hydrogen, but not at all or only poorly under anaerobic conditions produced by pyrogallic acid and alkali. Later an effort was made to try to explain the discrepancies that resulted from the use of these two methods of producing anaerobic conditions.

Exper. I.—Aerobic, facultative, partial tension, and anaerobic bacteria were grown under five different gaseous environments, produced as shown in the illustration. Equal inoculations from a suspension were always made.

1. Aerobic sealed. This tube contained O₂ and small quantities of CO₂ given off by the respiration of the bacteria.

2. Aerobic sealed with NaOH solution on the cotton plug. This tube contained O₂ and no CO₂.

3. Under H₂. This tube contained H₂ and small quantities of CO₂ given off by the bacteria.

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¹ Jour. Infect. Dis., 1918, 22, p. 194.

4. Under H_2 plus alkali. This tube contained no O_2 or CO_2 .

5. Under pyrogallie acid and alkali. This tube contained no O_2 or CO_2 .

The results of this experiment are given in table 1.

TABLE 1
RESULTS OF EXPER. 1

	Aerobic				Facultative				Partial Tension	Anaerobic	
	Hay Ba- cillus	Tu- bercle Ba- cillus 801	Tu- bercle Ba- cillus 802	Tu- bercle Ba- cillus 803	B. coli	Staph- ylo- coccus au- reus	Pro- teus vul- garis	B. an- thra- cis	Gono- coccus	B. welchii	B. te- tani
Aerobic sealed ($O_2 + CO_2$)	++ ++	++ ++	++ ++	+	++ ++	++ ++	++ ++	++ ++	+	—	—
Aerobic + alkali sealed (O_2 , no CO_2)	++ +	++ ++	++ ++	—	++ +	++ +	++ +	++ ++	+	—	—
H_2 (CO_2)	++ ++	++ +	++ +	—	++ ++	++ ++	++ +	++ ++	++ ++	++ +	++ —
H_2 + alkali (No O_2 , nor CO_2)	—	—	—	—	+	+	+	+	++	++ ++	+
Pyrogallie acid + alkali (No O_2 , nor CO_2)	—	—	—	—	+	+	+	+	—	++ ++	++ ++

In judging the amount of growth as indicated by + or —, the reading was taken in all instances in 24 hours, except in the case of 801 and 802 (48 hours) and 803 (two weeks). The medium used was beef infusion agar 1% acid to phenolphthalein; 6% glycerol was added to this medium for the growth of the saprophytic tubercle bacilli 801 and 802, while the virulent bovine strain 803 was grown on glycerol egg. Ascites fluid was added to the agar used for the growth of the gonococcus, *B. welchii* and *B. tetani*.

In table 1, among the so-called aerobic bacteria, the hay bacillus and the saprophytic tubercle bacilli, and the facultative bacteria, *B. coli*, *Staphylococcus aureus*, *B. proteus* and *B. anthracis*, all made their maximum growth under sealed aerobic conditions. The growth was somewhat arrested when the respiration CO_2 was removed by adding alkali to the aerobic condition; still less growth occurred under H_2 ; while under conditions furnished by H_2 and alkali, or by pyrogallie acid and alkali little or no growth occurred. The interesting point in this observation is that the aerobic and facultative bacteria would grow under H_2 , yet when alkali was added to the H_2 tube little or no growth

appeared. That is, when alkali was added to the hydrogen tube anaerobic conditions were produced which simulated that produced by pyrogalllic acid and alkali. Therefore it seems that the growth of aerobic and facultative bacteria are not only influenced by O_2 but also by CO_2 , as they are able to grow under O_2 alone, or $H_2 + CO_2$ alone, but they require one or the other or both of these gases. In other words, in producing anaerobic conditions two factors must be considered: first, oxygen removal, and second, carbon dioxide removal.

Strain 803 of the tubercle bacillus, a pathogenic bovine strain, grew only under sealed aerobic conditions, the removal of CO_2 preventing its growth. It seems therefore that a pathogenic tubercle bacillus requires both O_2 and CO_2 for growth, being unable to grow under O_2 alone or traces of CO_2 alone.

In the case of one partial tension strain tested, the gonococcus, growth occurred under all conditions except the anaerobic as produced by pyrogalllic acid and alkali; similar results were reported by Rockwell and McKhann.² Under H_2 the gonococcus made a very confluent growth; under H_2 and alkali a number of large colonies appeared, while under pyrogalllic acid and alkali no growth occurred. This shows that there is some difference between the three methods of producing anaerobic conditions, namely, displacement by H_2 , H_2 and alkali, and pyrogalllic acid and alkali.

It is evident that the chemical composition of the medium may decidedly influence the respiration of bacteria. This was clearly shown by the fact that when 1% glucose was added to the nutrient agar *B. coli*, *B. proteus vulgaris* and the hay bacillus grew equally well under H_2 , H_2 and alkali, and pyrogalllic acid and alkali, probably because the evolution of CO_2 exceeded its absorption.

It will be noted that in the case of the anaerobes, *B. tetani* grew poorly under H_2 , slightly better under H_2 and alkali and very well under pyrogalllic acid and alkali, again indicating that the three anaerobic methods employed produce different results.

B. welchii gave a better growth under H_2 the first 24 hours than under pyrogalllic acid, but in 60-72 hours the growth under the pyrogalllic method caught up and passed that under H_2 . This last observation was further supported by the following experiments:

Expt. 2.—*B. welchii* was grown under H_2 , $H_2 + 25\% CO_2$, $H_2 + 50\% CO_2$, $H_2 + 75\% CO_2$ and CO_2 . The result was that growth

² J. Infect. Dis., 1921, 28, p. 249.

occurred in every tube except the ones containing 75% or more of CO_2 , that is, a large excess of CO_2 interferes with its growth.

Exper. 3.—An equal inoculation of *B. welchii* was made into 5 tubes containing 5 c.c. of litmus milk. These were grown under various gaseous conditions. The gaseous environment and the results of two experiments are shown in table 2.

TABLE 2
RESULTS OF EXPER. 3

	Appearance of Stormy Fermentation in Hours	
	1	2
Aerobic sealed.....	30	—
Aerobic sealed + alkali.....	19	20
H_2	16	17
H_2 + alkali.....	15	16
Pyrogallie acid + alkali.....	15	16

It is apparent from this experiment that the gaseous environment influenced the period required for growth in milk and fermentation of milk by *B. welchii*, just the removal of CO_2 by alkali shortening the period considerably.

Judging by these experiments on anaerobes it is probable that CO_2 as well as O_2 influences their growth; further, it is again apparent that the anaerobic condition furnished by H_2 , H_2 and alkali, and pyrogallie acid and alkali, are not the same.

SUMMARY AND CONCLUSIONS

Having observed that some aerobic and facultative bacteria would grow under H_2 but not when pyrogallie acid and alkali were used to remove oxygen, further investigations were made. When planted on nutrient agar containing a trace of muscle sugar, the aerobic and facultative bacteria tested, two saprophytic tubercle bacilli, the hay bacillus, *B. coli*, staphylococcus aureus, proteus and *B. anthracis*, grew well in sealed aerobic cultures irrespective of whether the CO_2 produced by the bacteria was or was not absorbed by alkali. They also grew well under H_2 when the CO_2 produced by the bacteria was allowed to accumulate, but growth was arrested by placing alkali on the cotton plug of such H_2 cultures; nor did growth appear when pyrogallie acid and alkali were used. In other words, the growth of some bacteria ordinarily considered to be of the aerobic and of the facultative group, is in some way favored by CO_2 .

In the presence of glucose, *B. coli*, *B. proteus* and the hay bacillus grew equally well under H_2 , H_2 and alkali, and pyrogalllic acid and alkali, probably because the evolution of CO_2 exceeded its absorption.

In the case of a partial tension strain of the gonococcus, the best growth occurred under H_2 when the exhaled CO_2 was allowed to accumulate and was somewhat diminished by its removal; but no growth appeared in cultures made anaerobic by pyrogalllic acid and alkali.

In the case of the anaerobes, *B. welchii* and *B. tetani*, the growth of *B. welchi* was favored by the removal of the exhaled CO_2 ; *B. tetani* grew best when the O_2 and CO_2 were removed by pyrogalllic acid and alkali. It is not clear just why *B. tetani* failed to grow well under H_2 unless these cultures still contained small amounts of O_2 . If so, this would point to the inability of strict anaerobes to use even traces of atmospheric O_2 .

While many points concerning the influence, and the nature of the influence, of the gaseous environment on the growth of bacteria are still obscure, attention to the points brought out in this article may help in the isolation and differentiation of species.

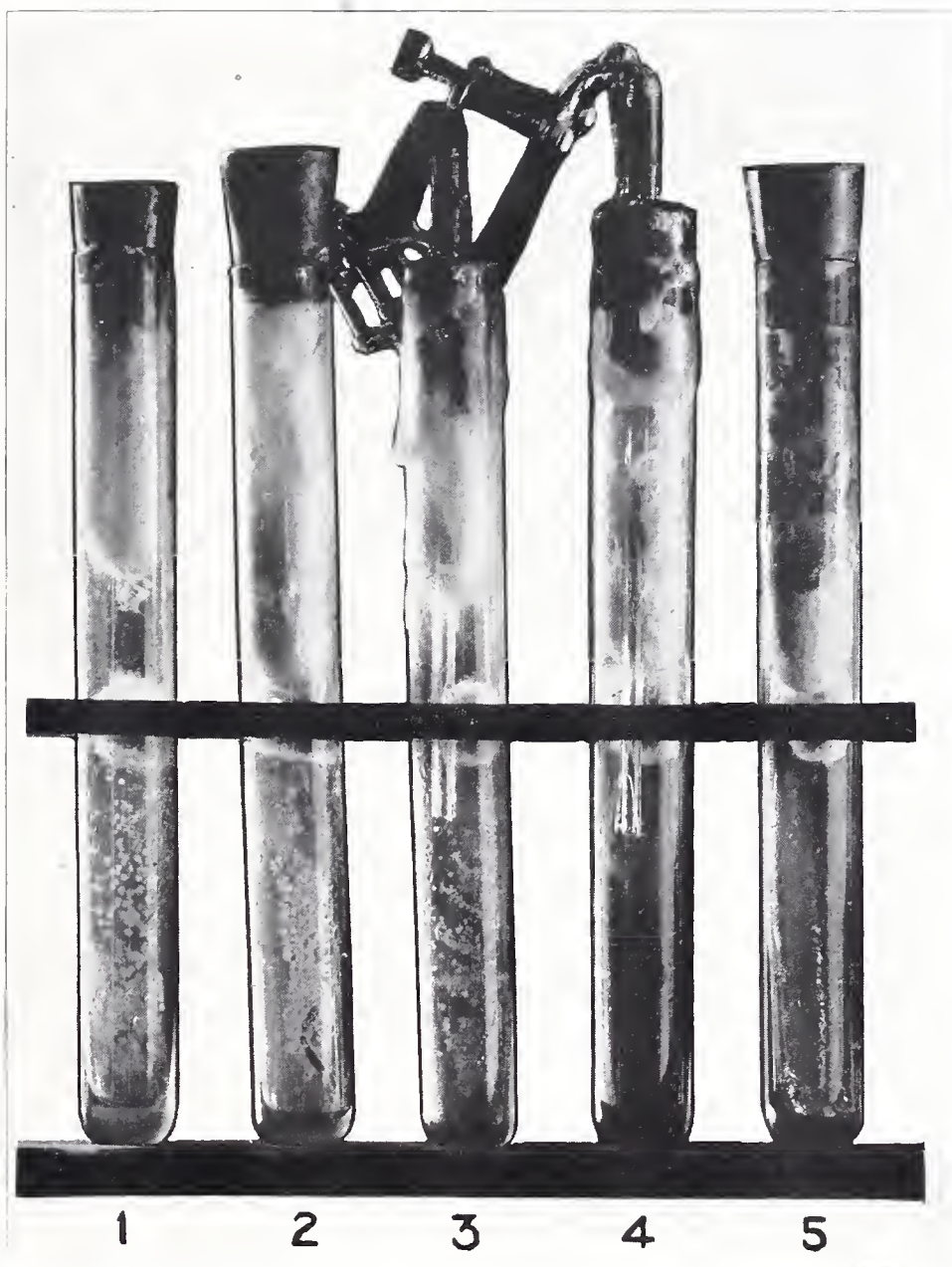


Fig. 1.—Photograph of the growth of *B. coli* as described in exper. 1. Tube 1 contains aerobic bacteria sealed; tube 2 contains aerobic sealed plus NaOH solution on the cotton plug; tube 3 shows anaerobic conditions produced by displacement with H_2 ; tube 4 shows anaerobic conditions produced by H_2 plus NaOH solution on a cotton plug; and tube 5 shows the anaerobic condition produced by pyrogallie acid and alkali. The method of displacing air with H_2 was the same as used by Rockwell and McKhann.²

INFLUENZA STUDIES

IV. EFFECT OF VACCINATION AGAINST INFLUENZA AND SOME OTHER RESPIRATORY INFECTIONS *

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In this study approximately 6,000 persons were under observation from November, 1919, to June 1, 1920. About one half of these were vaccinated with the bacterial suspension to be described presently; the other half were not vaccinated; both groups lived as far as might be under identical conditions.

Three schools and two large hospitals for mental diseases were available for these observations. The general physical condition of the subjects was good. Influenza vaccines had not previously been given; pneumonia vaccines, however, had been given in one school.

In the two hospitals at Kankakee and Jacksonville the candidates were selected from alphabetic lists. A certain number of patients showing signs of illness, including those having tuberculosis, were not considered suitable for observation. All the other inmates were listed alphabetically by wards. The subjects for vaccination were taken from these lists alternately, so far as the mental conditions of the patients permitted. Since the medical officers in charge thought the vaccine treatment undesirable in the management of some cases, certain objectors, whom it seemed unwise to coerce, were shifted to the unvaccinated side of the list. The number of such cases was comparatively small, and, as their objection consisted simply in unwillingness to have the hypodermic needle used, this shifting of cases did not affect materially the principle of arbitrary selection. It was unnecessary to discriminate in respect to seriously ill patients, as these had not been listed for observation and do not appear in either the "vaccinated" or "unvac-

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* This is one of a series of studies carried out in connection with the Influenza Commission established and financially aided by the Metropolitan Life Insurance Company of New York. Part of the expense of these studies has been met by a grant from the University of Chicago. In accordance with the plans drawn up by the Influenza Commission, this series of observations on the value of vaccination against influenza and other respiratory infections was carried out in certain public and private institutions of Illinois. We are under deep obligation to Mr. C. H. Thorne, Director of the Department of Public Welfare of the State of Illinois, and to Dr. C. St. Clair Drake of the Illinois State Board of Health, for their cordial cooperation in this undertaking. We are also indebted to the officials of the several institutions who have assisted us in every way.

inated" group. The ordinary fluctuations in the respiratory condition of the patients was not considered in the selection of subjects for vaccination.

The patients selected by the method of alphabetic alteration were brought in groups to the vaccinating staffs. They were successively inoculated by the institutions' physicians, and their names were then checked on the lists. At this point the schedule again received slight alterations, since some of the patients selected for vaccination could not be found at the time and were shifted to the unvaccinated group. In addition, there were a few minor oversights and misunderstandings, and in one or two wards the selections, while arbitrary, had not been in alternation from the lists.

A few patients who received the first dose but failed to appear for the second or for the third were dropped from the list altogether and are not included in the tables.

The result of all these minor changes is that the vaccinated group is somewhat smaller than the unvaccinated group, and the distribution of vaccinated cases, while mainly in accord with our original schedule, is not entirely so. It may, however, be stated that choice of the groups finally prepared for observation and comparison had not been influenced by any sort of purposeful selection other than, to some extent, the mental attitude toward injection.

It was not always possible to have in each ward equal numbers of vaccinated and unvaccinated, but the differences were not great. The Kankakee institution, for instance, supplied forty-one ward lists. The ward capacity varied from 15 to above 200 patients, being in very few instances below 30, in nearly half from 30 to 60 and in nearly half from 60 to 140. After all alterations of the lists, thirty-one of the ward lists indicated that in each ward from 46 to 52% of the patients available for comparisons had been vaccinated. Six of the remaining 10 lists showed from 24 to 41% vaccinated, 3 from 55 to 59% and one (with 32 patients) 100%.

In the schools for the blind and for the deaf similar alternate alphabetic selections were made from lists of pupils. In the third school, the University of Chicago, vaccination was optional.

All vaccinations were made with a saline suspension of a standard vaccine prepared under the direction of Dr. W. H. Park of the Influenza Commission in the Research Laboratory of the New York City Health Department. The vaccines were given subcutaneously in three doses at weekly intervals. The first dose contained: the Pfeiffer bacillus, 500

million; *Streptococcus hemolyticus*, 500 million; *Streptococcus viridans*, 500 million; pneumococcus type I, 1,000 million; pneumococcus type II, 1,000 million; pneumococcus type III, 500 million. The second and third doses contained double these numbers of each organism. A few of the smaller children were given only half doses. The injections were made between November 24 and December 11 in 4 of the institutions, and between November 3 and December 16 in the fifth (the University).

The Kankakee and Jacksonville State Hospitals for the Insane included respectively 3,012 and 2,145 cases available for comparison. Practically all were adults. The general condition of these patients was for the most part good. In recent years perhaps a dozen pneumonia cases have developed yearly in each institution. The Kankakee Hospital was touched very lightly by the influenza epidemic of 1918-19. About 12 pneumonia cases developed, hardly more than in normal years. Approximately 100 cases were then diagnosed as influenza, many of these diagnoses doubtless being influenced here as elsewhere by the general prevalence of the disease.

The Jacksonville Hospital, on the other hand, suffered from a pronounced influenza epidemic in the fall of 1918. The Managing Officer's report of Dec. 3, 1918, shows a record of 358 influenza cases, with 17 deaths. This institution was quarantined Oct. 1, 1918; the first cases developed October 16, the largest number October 28 and the last on November 25. None of the patients at that time received a prophylactic vaccine against influenza or pneumonia.

The State Schools for the Deaf and for the Blind, both at Jacksonville, included respectively 355 and 207 cases for comparison, all the patients being children. The School for the Blind escaped the influenza epidemics of 1918-19, but the School for the Deaf had a marked outbreak of influenza during that period. About 150 influenza cases developed in the latter institution, many being complicated by pneumonia. There was one death. Some of the pupils in this school had previously received "antipneumonia" vaccines of unspecified composition.

Approximately 600 students of the University of Chicago volunteered to cooperate in a respiratory disease study. A number of these gave a detailed preliminary report covering history, susceptibility, etc., and followed this up by reports of respiratory disorders as they occurred, with a final report on June 1, 1920. Vaccination was given

to those who desired it. It is evident that grouping according to voluntary application for vaccination is unsatisfactory as compared with arbitrary alternate selection from alphabetic lists, as was done in the other 4 institutions; 164 students received the three vaccine injections and 183 none. Some students that had previously received respiratory vaccines are not included in this summary, nor are any except those whose reports covered the entire period of observation.

Those persons who received only one or two doses of the vaccines are excluded from the series. The figures for the various institutions comprise only persons included in our lists who received either three injections or none.

Reports of all respiratory ailments developing in the University group were made by the students themselves on printed forms, and were supplemented when practicable by the observations of a medical examiner. In the state institutions the respective medical staffs observed, diagnosed and recorded such conditions without respect to the groups involved. These records were sent to us each month, for comparison with our original lists of persons observed.

When the influenza epidemics of January and February, 1920, spread over Illinois, the 5 institutions under observation were all touched. It is interesting to note that so far as could be determined the distribution among these institutions was about proportional with the distribution of the previous epidemics of 1918-1919. The Jacksonville Hospital alone had a considerable number of cases, and it did not suffer heavily.

The city of Jacksonville had 1,000 or so influenza cases. The Jacksonville State Hospital developed 172 and the State Schools for the Deaf and for the Blind 20 and 13, respectively. The symptoms were typical; chilliness at onset, cough, headache and sometimes general aching, a temperature of 101 to 104 degrees, down after 2 days or sometimes irregular for a few days more, etc. Complicating pneumonia was usually of the bronchial type.

While the city of Kankakee had a marked epidemic, patients of the Kankakee State Hospital escaped with 18 cases. The University of Chicago group reported 47. Our figures are somewhat below the total incidence in the institutions by reason of certain exclusions from our lists as elsewhere explained.

Table 1 shows the effect of the prophylactic vaccinations on the development of influenza. The total number of observed persons is

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seen to be 47.4% vaccinated and 52.6% not vaccinated. Of the vaccinated groups 118 (4.1%) contracted influenza, 7 (5.9%) of whom developed pneumonia, with two deaths. Of the unvaccinated groups, 152 (4.8%) contracted influenza, 12 (7.9%) of whom developed pneumonia, with two deaths. These figures do not indicate that any considerable degree of immunity is conferred by the vaccine; the number of cases is too small to warrant attaching much importance to the slight difference observed. No one age group is better protected than another. This is likewise true of the other pneumonia findings to be presented.

TABLE 1

COMPARISON OF INFLUENZA AND INFLUENZAL PNEUMONIA RATES AMONG THE VACCINATED AND THE UNVACCINATED

Age Group	Vaccinated						Not Vaccinated					
	Influenza Cases					Pneumonia Complications, All Five Institutions	Influenza Cases					Pneumonia Complications, All Five Institutions
	Kankakee	Jacksonville	Deaf School	Blind School	Chicago		Kankakee	Jacksonville	Deaf School	Blind School	Chicago	
5-10	0	0	4	0	0	0	0	0	0	0	0	0
11-15	0	0	3	3	0	0	0	1	5	2	0	0
16-20	0	1	2	3	3	0	0	1	3	1	12	3
21-25	1	3	0	2	12	0	1	4	0	1	9	0
26-30	0	6	0	1	1	1	0	8	0	0	3	0
31-35	0	4	0	0	3	0	3	4	0	0	1	0
36-40	0	11	0	0	1	1	2	18	0	0	0	3
41-45	1	6	0	0	1	0	0	10	0	0	0	1†
46-50	0	9	0	0	0	0	0	20	0	0	0	3†
51-55	3	9	0	0	0	3†	2	5	0	0	0	0
56-60	1	6	0	0	0	0	2	11	0	0	0	1
61-65	1	5	0	0	0	0	0	10	0	0	0	0
66-70	0	5	0	0	0	0	0	6	0	0	0	1
Over 70	0	5	0	0	0	1†	0	3	0	0	1	0
Unk'wn	0	0	2	0	0	1	1	0	1	0	0	0
Totals	7	70	11	9	21		11	101	9	4	27	
Total influenza..... 118							152					
Pneumonia complications.....						7†						12†

	Total Number Under Observation*	
	Vaccinated	Not Vaccinated
Kankakee.....	1,457	1,555
Jacksonville.....	968	1,177
Deaf School.....	182	173
Blind School.....	102	105
University of Chicago.....	164	183
	2,873	3,193

* The small attack rates seemed not to justify the clerical work of age-grouping all observed persons.

† Of the influenzal pneumonia cases, 4 died, 2 among the vaccinated and 2 among those not vaccinated, 1 in each age group indicated.

It seemed possible that disease incidence or prophylactic reaction might be different among the new arrivals as compared with the old residents. Table 2 enumerates cases selected from table 1 including patients who had been admitted to the residence institutions during the year preceding our inoculations. The influenza incidence is seen to be higher than for the total groups (vaccinated 6.0% and unvaccinated 6.9%) and the pneumonia complications lower (one case unvaccinated), but the proportions between vaccinated and unvaccinated are approximately equal.

TABLE 2
INFLUENZA RATES * AMONG PERSONS ADMITTED WITHIN THE YEAR PRECEDING THE
INOCULATIONS

Age Groups	Vaccinated				Not Vaccinated			
	Kanka- kee	Jack- sonville	Deaf School	Blind School	Kanka- kee	Jack- sonville	Deaf School	Blind School
Under 21.....	0	1	2	0	0	1	2	0
21-25.....	1	1	0	1	0	2	0	0
26-30.....	0	3	0	0	0	4	0	0
31-35.....	0	2	0	0	1	0	0	0
36-40.....	0	4	0	0	1	4	0	0
41-45.....	1	1	0	0	0	3	0	0
46-50.....	0	4	0	0	0	5	0	0
51-55.....	0	0	0	0	1	1	0	0
56-60.....	1	2	0	0	1	3*	0	0
Over 60.....	0	1	0	0	0	3	0	0
Influenza totals.....	3	19	2	1	4	26	2	0
Total observed.....	215	151	40	10	265	171	11	16
Grand totals.....	25 cases among 416 persons observed				32 cases among 463 persons observed			

* Of this series, but one case (age 56 and not vaccinated) developed a complicating pneumonia.

Small numbers are particularly unreliable for conclusions on influenza because diagnosis is so largely influenced by epidemic conditions. The Jacksonville Hospital, which suffered the disease in epidemic proportions, might presumably give a truer index than the 5 institutions combined. In that institution the vaccinated patients had an influenza incidence of 7.3%, the unvaccinated 8.6%, proportions not unlike the totals already given.

The observed pneumonia cases not associated with influenza are likewise few in number. Practically all of them occurred in the hospitals at Kankakee and Jacksonville. Nineteen cases developed in these institutions, 6 among the vaccinated and 13 among the unvaccinated. Twelve lobar pneumonia cases occurred, 5 with 3 deaths among the vaccinated and 7 with 4 deaths among the unvaccinated. Tuberculous

pneumonias are not listed. Of cases reported as bronchopneumonia (not during the influenza epidemic) or as pneumonia, type unspecified, there were among the vaccinated 1 and among the unvaccinated 6, with 2 deaths. These proportions, although the actual numbers are small, suggest some degree of immunity to pneumonia due to vaccination. It may be noted that the balance in favor of pneumonia prevention is from the Jacksonville list, the Kankakee cases being about equally divided.

It seemed worth while to determine whether the vaccines used protected against attacks of ordinary colds. The three schools with 909 controlled patients give some information on this point. The greater part of the reports received do not differentiate between various types of colds sufficiently to allow any but collective grouping of such conditions.

Table 3 shows that the vaccinated group, although slightly smaller, reported a somewhat greater total number of colds, and of persons affected with colds than the unvaccinated. This may be misleading, since those who are voluntarily vaccinated are quite likely to be more inclined to notice and report colds carefully. Age groups up to 30 years are included. The figures indicate that such vaccines do not influence in any noteworthy degree susceptibility to ordinary colds.

The proportion of colds reported from the two hospitals was very small, as might be expected from the difficulty of observing such minor ailments among persons with mental diseases. There were reported from the Kankakee and Jacksonville Hospitals, respectively, 135 and 32 colds among the vaccinated and 112 and 17 among the unvaccinated.

Only the University reports are such as to differentiate reliably nose from chest colds. Table 4 shows that the vaccinated group of 164 developed 248 rhinitis attacks, 26 bronchitis and 44 of rhinitis and bronchitis combined, while the unvaccinated 183 developed 216 rhinitis, 25 bronchitis and 28 combined attacks. These figures indicate that no greater protection is obtained against either variety of cold than against the collective average.

As suggested in the foregoing, the voluntary choice of vaccination by the University students admits a possible error. The more susceptible might desire and the less susceptible might be less desirous of prophylactic inoculations. We therefore attempted to determine whether the yearly average of colds in either group was altered following the injections. At the beginning of the observational period, each

TABLE 3
RATE OF ORDINARY "COLDS" AMONG VACCINATED AND UNVACCINATED GROUPS

Age Groups	Vaccinated										Not Vaccinated									
	Persons Under Observation					Persons Contracting Colds					Persons Under Observation					Persons Contracting Colds				
	Chi- cago	Deaf School	Blind School	Number of Colds Con- tracted		Chi- cago	Deaf School	Blind School	Number of Colds Con- tracted		Chi- cago	Deaf School	Blind School	Number of Colds Con- tracted		Chi- cago	Deaf School	Blind School	Number of Colds Con- tracted	
5-10.....	0	55	21	0	52	0	30	5	0	7	0	31	21	0	2	0	17	2	30	2
11-15.....	0	83	37	0	50	0	30	4	0	4	2	74	42	1	5	1	32	5	57	6
16-20.....	45	39	34	43	17	99	14	7	10	10	92	64	22	78	21	138	21	4	28	4
21-25.....	90	3	8	3	3	162	2	2	3	3	51	3	13	41	0	77	0	0	0	3
26-30.....	17	0	2	17	0	30	0	0	0	0	22	0	7	20	0	34	1	0	0	2
Over 30 or unknown.....	12	9	0	11	0	27	0	0	0	0	16	1	0	14	0	19	0	0	0	0
Totals.....	164	182	102	132	122	318	76	18	24	24	183	173	105	154	70	269	115	17	461	401
Under observation.....	448					246					461					238				
Contracted colds.....				
Number of colds.....				

student recorded the average number of colds suffered yearly. Later, reports were obtained showing how many colds developed during this period. The latter reports cover the more prolific respiratory disease months but not an entire year; we might therefore expect fewer colds than the yearly average. Of the vaccinated (table 5), 73 had their usual yearly average of colds, 39 had more and 52 less. Of the unvaccinated, 77 had their usual yearly average, 44 more and 62 less. These figures, showing that the vaccinated persons experienced no greater reduction in the liability to colds than the unvaccinated, largely discount the possibility of error just mentioned.

TABLE 4
ATTACK RATES OF RHINITIS, BRONCHITIS AND THESE COMBINED
(University of Chicago)

Age Groups	Attacks Among the Vaccinated, of			Attacks Among Those Not Vaccinated, of		
	Rhinitis	Bronchitis	Rhinitis and Bronchitis	Rhinitis	Bronchitis	Rhinitis and Bronchitis
11-15.....	0	0	0	0	1	0
16-20.....	73	12	14	113	10	15
21-25.....	135	7	20	62	9	6
26-30.....	22	1	7	30	1	3
Over 30 or unknown.	18	6	3	11	4	4
Totals.....	248	26	44	216	25	28

TABLE 5
FREQUENCY OF COLDS AS AFFECTED BY THE INOCULATIONS
(University of Chicago)

Age Groups	Vaccinated Persons With			Persons Not Vaccinated With		
	More Colds Than Usual	Fewer Than Usual	Usual Number of Colds	More Colds Than Usual	Fewer Than Usual	Usual Number of Colds
11-15.....	0	0	0	1	1	0
16-20.....	11	6	28	20	36	36
21-25.....	21	36	33	14	15	22
26-30.....	3	5	9	5	2	15
Over 30 or unknown.	4	5	3	4	8	4
Totals.....	39	52	73	44	62	77

The reports of other respiratory disorders, tonsillitis, pharyngitis, "sore throat" and laryngitis are too few to warrant any definite conclusions. Both vaccinated and unvaccinated groups, however, reported substantially the same number and kinds of ailments.

An interesting side-light on the subjective reaction to vaccination is furnished by perhaps a dozen unsolicited testimonials. Those vaccinated at the University had been told that possibly colds might be

prevented by vaccination, and toward the end of the period many were kind enough to express their opinion of the result. Two thought the vaccine had done them no good, but most of them optimistically declared that they had received definite benefit in protection against colds. "Satisfied patient" conclusions differ widely from those of controlled statistics.

SUMMARY

The prophylactic effect of a widely used vaccine containing Pfeiffer bacilli, streptococci and pneumococci has been studied clinically and statistically. We have recorded during a period of about 7 months the respiratory ailments which developed among 6,066 persons, approximately half of whom had received the vaccine. Some of these were attacked by influenza in the 1920 wave, which occurred within two months of the vaccination; in addition, the usual number of pneumonia and common cold cases among those observed afford material for comparisons.

Rhinitis and bronchitis developed with frequency about equal in vaccinated and unvaccinated groups.

The influenza attacks among the 2,873 vaccinated numbered 118 (4.1%) and among the 3,193 unvaccinated numbered 152 (4.8%); 7 pneumonia complications with 2 deaths occurred among the 118 vaccinated patients and 12 with 2 deaths in the 152 unvaccinated. Both the influenza and pneumonia attack rates are hence somewhat lower among the vaccinated, but the difference is not great. Pneumonia, not associated with influenza, was also less frequent among the vaccinated, only 6 of 19 pneumonia patients having been vaccinated. The small numbers hardly warrant, although they suggest, a favorable conclusion regarding some slight prophylactic value for pneumonia. That any considerable degree of protection against influenza was conferred by the vaccine seems unlikely.

A NEW METHOD OF ADDING CRESOL TO ANTI-TOXINS AND ANTISERUMS

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As is well known, the addition of cresol to antitoxins and other antiserums results in the formation of a precipitate due to the combination of the cresol with the proteins. This precipitate is greatest when all the cresol needed to give the desired concentration is added rapidly. To avoid this more or less voluminous precipitate, various methods have been resorted to. The cresol may be added in a dilute form. This method has the disadvantage of diluting the product to which it is added. Another method is to add the undiluted cresol drop by drop to the product. In this method the serum is commonly placed in an open vessel and beaten with a rod or paddle as the cresol is added to obtain thorough mixing. Similarly, admixture is often accomplished by adding the cresol drop by drop to the large stock bottles, replacing the cork and shaking the container thoroughly after each addition. These methods are not above reproach, as they open the way to contamination. Different producers have devised various methods to limit the exposure to contamination during the addition of the preservative. It is evidently essential that the amount of precipitate formed should be limited as much as possible. In the first place, the precipitate binds up the preservative and thus lessens the amount available throughout the product. How much remains in the serum is not known. Furthermore, the precipitate increases the loss of the product in the process of filtration and adds as well to the difficulty of this process.

These considerations raised the question whether we could not find a better method for the addition of cresols to antitoxins and serums. We considered the possibility of utilizing stable emulsions using menstrua other than water, in which emulsions separate quickly. Solvents other than water in which the solubility of cresol is low, were also considered. After several preliminary experiments, ether was tried as a solvent. It was found that equal parts of cresol and ether are miscible and make a permanently clear solution. On the addition of such a mixture to antitoxin or serum, the solution floats on the

surface causing a slight haze at the point of contact. If shaken immediately after the addition either no precipitate or at most very little is formed. Even the rapid addition of all the solution needed to give a concentration of 0.4% to 0.5% of cresol, causes at most only a slight precipitation; the amount of which is in no way comparable to that resulting from the rapid or even drop by drop addition of the same amount of cresol alone.

With this success in eliminating precipitation when the whole volume of preservative was quickly added, several questions immediately arose: First, what would be the effect of the additional ether when the product is administered? Second, would the product, on aging, precipitate, or in other words would the cresol slowly but eventually cause as much precipitate as if it alone had been added at first? Third, would the loss of ether during filtration and other processes result in objectionable precipitate? Fourth, would the bactericidal action of cresol be decreased by the ether or not? If not, would the additional bactericidal action of the ether be of value? Fifth, would the ether affect the potency of the product?

We are indebted to Dr. Gwathmy for an opinion as to the probable harmlessness of the ether, in the amounts under consideration, when given intraspinaly or intravenously. The intraspinal injection of antimeningococcus serum with the ether-cresol preservative was first tried with a monkey. As there was no evidence of pain or untoward after effects, the product was then tried in cases of cerebrospinal meningitis by Dr. Josephine Neal and her associates. They could see no difference between the serum thus preserved and that containing cresol only.

We have been unable thus far to obtain similar trials of the intravenous injection of antipneumococcus serum with the combined preservative. However, Dr. Russell Cecil has added 0.4 c c of ether per dose of antibody solution of Huntoon and finds no evident ether effect, when this is given intravenously. We see no reason, therefore, for hesitancy in using serum containing the combined preservative. At the Willard Parker Hospital, diphtheria antitoxin containing this preservative has been utilized for intravenous injections. With the doses employed, the amount of ether is small and no ill effect is noted. One of us (E. J. B.) had observed, in conjunction with Dr. C. A. Thompson of the hospital staff, that chilliness or actual chills were more common when the intravenously administered antitoxin showed cloudiness or a

slight precipitate. For the last few months a preparation to which ether-cresol had been added has been in use and although this preparation is now cloudy chills are not induced. Another preparation, however, containing cresol only, but which is very clear, has just been tried and causes chills. The addition of 0.4% of ether to this product does not lessen these reactions. The conditions in these two preparations were not quite the same, as in the second preparation the ether was added separately. These experiences would seem to suggest that ether, under certain conditions may influence such reactions. A further study of this subject under comparable conditions seems indicated.

Experience shows that the subcutaneous injection of antitoxins containing ether-cresol are not rendered more painful by the presence of the ether. The use of ether in hypodermic therapy would lead one to anticipate this.

As to the subsequent development of precipitation in antitoxins and serums to which ether-cresol has been added, we can at least say that it is no greater than had cresol been added alone. In the case of antitoxic globulins, an example may be cited of a lot taken from the dialyzing bags and filtered through paper pulp to clarify it. Ether-cresol was then added. At most, only a slight opalescence developed and this did not increase nor did an appreciable precipitate form even after several months in the ice chest.

To test the effect on serums, a bleeding was taken and split into three parts. To one part was added cresol, to another, cresol-ether, and no preservative to the third. The following tabular summary gives the degrees of precipitation observed in samples filtered at increasing intervals after the addition of the preservative.

Serum. Preservative added 3/13/20.

Successive Samples, Filtered	Relative Amount of Precipitate 11/5/20		
	Cresol	Cresol-Ether	No Preservative
4/ 9/20	+	+	++
4/23/20	+	+	(not done)
6/ 3/20	+	±	++
10/30/20	—	—	Trace

Symbols ± to ++, relative amounts of precipitate; —, none.

In these filtered samples, the precipitation progressed about equally in the cresol and ether-cresol samples but more rapidly in the sample without preservative. The last was not due to bacterial contamination as was shown by cultural tests.

In the original unfiltered samples there was a copious precipitate in the sample without preservative, about half this amount of precipitate in the sample containing cresol alone. The sample containing ether-cresol had less than one-tenth the precipitate present in the cresol sample. This would be expected, as the precipitate caused by the addition of the cresol would be present in the cresol sample, as well as the precipitate which subsequently formed. It is of interest to note that the greatest precipitation on aging was encountered with the serum containing no preservative.

With two other samples of serum to which the preservatives were added on March 17, 1920, and which were filtered April 15, 1920, the precipitation observed Nov. 5, 1920, was more marked in the case of the sample to which the cresol alone was added.

The amount of ether lost during the processes of filtration, that is, filtration through paper pulp and through the Berkefeld filter, has not resulted in the formation of a precipitate during the stages of the process or as it finally comes from the Berkefeld filter. The filtered product is crystal clear. The amount of ether is small and is most probably in true solution. There can be little doubt of this as ether is soluble in water up to practically 10%. We have gained the impression that when serum is saturated with ether evaporation of the ether becomes progressively more difficult as the percentage left decreases. Even prolonged heating will not rid the serum of the ether odor. If this impression is correct, it would indicate that of the slight amount (0.4%) added to the serum, little is lost during the process of filtration.

In regard to the fourth question, namely, would the ether lessen the bactericidal action of the cresol or would it leave it unchanged, and if the action of the cresol were unchanged, would the bactericidal action of the ether make the mixture appreciably more active, the appended tables of the disinfectant tests show that the mixture is more efficacious than cresol alone.

We expected this because of some previous experiences with tetanus antitoxin. We have from time to time, as have others, encountered antitoxins and other products which are contaminated with a cresol resistant bacterium (that is, one resistant to the amount of cresol used). This bacterium passed through the filter in small numbers so that the sterility tests immediately after filtration often failed to reveal their presence. After several weeks' storage, however, the product

would again reveal this contaminant due apparently to some multiplication of the bacterium in spite of the cresol.

TABLE 1
COMPARATIVE BACTERICIDAL ACTION OF CRESOL AND OF CRESOL DISSOLVED IN ETHER
Tested 2/26/20

Sample	Dilution*	Time of Exposure		
		2½ Minutes	5 Minutes	7½ Minutes
Cresol	1:200	—	—	—
	1:250	—	—	—
	1:300	+	+	—
Cresol dissolved in ether	1:200	—	—	—
	1:250	—	—	—
	1:300	+	—	—

Hygienic Laboratory Standard Method.

— = no growth, + = growth, 48 hrs. 37 C. readings.

* The dilutions refer to the dilution of the cresol. As the cresol was dissolved in an equal quantity of ether, the dilutions under "cresol dissolved in ether" indicate also the dilution of the ether.

TABLE 2
COMPARATIVE BACTERICIDAL ACTION OF CRESOL AND OF CRESOL DISSOLVED IN ETHER
Tested 2/28/20

Sample	Dilution	Time of Exposure		
		2½ Minutes	5 Minutes	7½ Minutes
Cresol	1:200	—	—	—
	1:250	+	—	—
	1:300	+	—	—
Cresol dissolved in ether	1:200	—	—	—
	1:250	—	—	—
	1:300	—	—	—

Hygienic Laboratory Standard Method.

— = no growth, + = growth, 48 hrs. readings.

In several instances during the war, the shortage due to this type of contamination became so acute due to the unprecedented demands that we resorted to the addition of ether. We did this in the light of the more or less general use of this method to sterilize albuminous fluids for bacteriological use. The addition of the ether quickly sterilized the antitoxin. In these instances, a proportionately large amount of ether was employed and the excess was subsequently driven off by heat.

More recently we encountered a similar contamination in a lot of toxin-antitoxin mixture. The addition of only 0.4% of ether resulted in apparent sterilization. The tests of sterility were not made until seven and eight days after the addition of the ether so that we do not

know how soon sterilization was effected. This amount of ether (0.4%) has, however, failed to sterilize one lot of contaminated tetanus antitoxin.

On the whole, our experience and that of others who have tried the combined ether-cresol preservative, indicates that the increased bactericidal activity is of decided practical importance, especially in relation to those products which, because of their mode of preparation, cannot be kept sterile.

Experience has shown that the potency of serums and antitoxins is uninfluenced by the ether. Preparations of diphtheria antitoxin, antimeningococcus serum and of antipneumococcus serum have been retested as long as 7 months after addition of the ether cresol. The ether may be added to a toxin-antitoxin mixture without interfering with the balance as the ether is without effect on the more labile toxin element. Thus on Aug. 27, 0.4% of ether was added to a toxin-antitoxin mixture. This mixture was toxic, killing guinea-pigs on the fifth or sixth day. Tested 53 days later, the pigs died on the sixth day.

Certain theoretical considerations have arisen while collecting the purely practical facts outlined in the foregoing. These points may be mentioned. On mixing the ether and cresol, considerable heat is generated. We have not attempted to determine whether any new compound is formed. If the mixture consists of 25% of ether and 75% of cresol, instead of half and half, the addition to serum results in a marked opalescence of the serum although marked precipitation may not occur. Ether tends to reduce the surface tension of the serum. This may possibly be a factor not only in inhibiting precipitation but also a factor in the apparent inhibition of reaction after the intravenous administration of antitoxin. We have not attempted to give a summary of the previous use of ether as a preservative nor of the use of ether in the prevention of anaphylactic shock in experimental animals. The former is generally well known. The latter would involve a discussion which we wish to avoid by simply presenting the practical results obtained without any attempt at explanation.

SUMMARY

A mixture of equal parts of ether and cresol is presented as a new preservative for antitoxins and serums. This mixture is added in amounts necessary to give the required concentration of cresol. The addition of this mixture causes much less precipitate than does cresol

alone. Subsequent precipitation is not necessarily limited by the ether, it is never greater than that in products containing cresol alone. The mixture of ether and cresol is more strongly antiseptic than cresol alone. In therapeutic application, the ether is not a disadvantage. In the case of intravenously injected antitoxin, the indication that the ether may under certain circumstances reduce the incidence of adverse reactions warrants further comparative work. Ether may be added to the toxin-antitoxin mixture without disturbing the balance of the mixture.

THE CORRELATION OF RABBIT PNEUMONIA AND HUMAN INFLUENZAL PNEUMONIA

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In this paper I wish to report certain studies on pneumonia in rabbits and incidently to compare the lesions with the lesions in human influenzal pneumonia.

An epidemic of rabbit influenza occurred among the laboratory animals in the fall and winter of 1918 and the spring and summer of 1919. While contemporaneous with the epidemic of influenza, there was no reason whatever to surmise a common etiology. It had occurred many times before in the laboratory.

The duration of the disease was variable; most animals succumbed at the end of from 5 to 7 days. Early there was loss of appetite and a thin, watery nasal discharge, accompanied by frequent sneezing. The discharge descended from the nares to the breast and anterior extremities and, as the disease progressed, became mucoid and purulent in character. Animals used for experimental purposes (infections, vaccine injections, etc.) appeared more susceptible and succumbed 6 to 48 hours earlier as a rule.

The exciting cause is the *B. bipolaris*, an organism classed in the hemorrhagic septicemia group. This organism has been reported under various names: e. g., *B. bronchisepticus*, *B. bovissepticus*, *Bacillus of pleuropneumonia*, *Bacillus of rabbit septicemia*, etc. In this article I use the term *B. bipolaris*. It was recovered from the following sources: nasal discharge, nasopharynx, pleural fluid, pericardial fluid and heart blood. Koch's postulates were fulfilled. The bacillus is short, about 1-3 microns in length, staining intensely at the poles and only slightly in the middle, and at times is pleomorphic. It is nonmotile, nonspore-forming, aerobic and facultative anaerobic. It stains with the ordinary aniline dyes and is negative to the Gram stain. In fluid medium the organism is often coccoid while on solid medium it tends to retain its characteristic form. It has appeared in cultures as a coccoid bacillus, a diplobacillus, a streptobacillus and at times in threadlike

forms similar to *B. influenzae*. After cultivation on artificial medium for a few generations, it tends to lose its characteristic bipolar staining.

In the literature are reports of a number of varieties of bacilli isolated from rabbits dying of lung involvement. Beck¹ mentions a small gram-negative, nonmotile bacillus, pathogenic for rabbits, guinea-pigs and mice, having a marked tendency to form threads, as the cause of "Breustseuche" in rabbits. Laven² described a bacillus pathogenic for rabbits and guinea-pigs; small, gram-negative, variable in size and a tendency to grow in thread and chain forms. It is strictly hemolytic and gives a peculiar sperm-like odor on blood agar. Kurita³ described a small gram-negative polar staining bacillus which killed animals when injected by producing "Breustseuche." Undoubtedly the organism isolated from this series of cases belong to the same general group as those above described. It produces a confluent lobular pneumonia on intra-tracheal insufflation.

Experimental production of the disease was effected as follows: a 24-hour agar slant of bacteria was emulsified in 5 c.c. sterile normal salt solution and sprayed into the nose and nasopharynx of 5 healthy animals. All succumbed to the disease in the acute stages. To prevent the possibility of contamination at the institution, 2 animals, obtained from a source other than that from which they were usually obtained and kept at a distance of some miles from the laboratory, readily contracted and succumbed to the disease 8 days after inoculation.

A series of 17 animals was collected, of which 12 succumbed to the disease acquired in the natural manner and in 5 the disease was produced experimentally. Necropsies were performed within 1 to 14 hours after death. Sections for microscopic study were taken from the lungs, trachea, heart, liver, kidney and spleen, fixed in formalin or Zenker's fluid, sectioned in paraffin and stained with hematoxylin and eosin. In certain instances, special staining methods were also used.

Macroscopically the lungs presented the typical picture of a confluent bronchopneumonia. They did not completely collapse on opening the chest and the pleural surfaces were frequently mottled with patchy areas of dark, bluish red color; often in the acute stage the pleura was covered with a thin layer of fibrin. The pleural cavities usually contained some fluid. Small consolidated masses varying in size from that of a pinpoint to that of a pea, hard and firm, with crepitating lung tissue surrounding them could be felt, usually more prominent in the lower lobes and especially of the right lung. The distribution of the bronchopneumonic areas was as follows: upper lobe, left 12%, right 14%; middle lobe, right 18%; lower lobe, left 26%; right 30%.

On cut section the lungs were moist and edematous, with a red, frothy liquid, sometimes purulent in character, exuding from the cut ends of the bronchioles. On scraping, in some instances, plugs of necrotic material were removed. The distribution of the consolidated areas was variable; in the more

¹ Kolle-Wassermann: Handbuch der path. Mikroorg., 1903, 3, p. 405.

² Centralbl. f. Bakteriol., 1, O., 1910, 54, p. 97.

³ Ibid., 1909, 49, p. 508.

acute cases it was usually in small patches near the periphery of the lung, in the more chronic it often became confluent in character and was situated around a central bronchiole. The consolidated areas were usually surrounded by a zone of hyperemia. At times the centers of these consolidated areas appeared necrotic. Engorgement of all the pulmonary vessels was particularly evident.

Microscopically, the picture varied with the stage of the disease. In the acute stage, perivascular edema and leukocytic infiltration were the most prominent features. In some instances, a clear, edematous exudate containing few or no cells was prominent. In rabbit 6, there was a proliferation of cells beneath the intimal coat of the blood vessels. As the stage of the disease became more advanced, it tended to resemble red hepatization. The alveolar epithelial cells were swollen, edematous and degenerative. In some instances, complete desquamation had taken place. The interstitial tissue was edematous and swollen, the vessels hyperemic and distended, with perivascular infiltration, as a rule. The alveoli were packed with red cells and desquamated alveolar cells together with strands of fibrin interspersed. In the advanced stage, the invasion of large numbers of white cells occurred, similar to gray hepatization. The fibrin increased in amount and later at times became organized. The outlines of the alveoli were indistinct and in some areas imperceptible, the process having become confluent with subsequent obliteration of the individual alveoli. Occasionally the pneumonic process was complicated by a tendency to small abscess formation. In the small consolidated areas, central necrosis, quite intense in some instances, was noted. Not infrequently this process involved over one half of the consolidated area. In many instances the bronchioles contained an exudate, the constituents of which depended on the stage of the process. All stages of cellular degeneration could be observed. Phagocytosis was often seen; the phagocytized structures being red cells, polymorphonuclears and cellular debris. Definite focal hemorrhages were occasionally seen, often involving large areas.

In the acute cases, less alteration was noticed, a slight transudation of serum and a few leukocytes plus slight hyperemia being the chief manifestations. In the more subacute and protracted cases, the picture varied from degenerative changes of the epithelial lining to complete desquamation and necrosis of the underlying tissue. The larger bronchi were less involved, this varying from slight to intense necrosis and sloughing. In most instances the peribronchial lymphatic spaces were distended and infiltrated with leukocytes.

The trachea contained a slimy, mucoid or frothy, slightly blood tinged fluid, especially near the bifurcation. On removal of this material, intense hyperemia of the mucosa was evident. In some instances, on opening the trachea, the affected side revealed more of the frothy, blood tinged fluid showing a fairly sharp line separating the diseased from the normal side. Microscopically an acute tracheitis was present, with edema and marked degenerative changes in the epithelial lining in some instances; in others only a moderate hyperemia was present.

Desquamative bronchiolitis was frequent in places with complete or nearly total destruction of the mucosa. Strands of fibrin and polymorphonuclear cells were found abundantly in some bronchioles; others appeared quite normal.

The heart was little altered. In most cases, a few c.c. of clear, straw-colored fluid was contained within the pericardial sac. In no animal in this series was there evidence of a fibrinous pericarditis. Occasionally the right heart was dilated. The valves were normal in every case except one in which a small vegetation occurred on the mitral valve and endocardium, from which

a mixed culture of hemolytic streptococci and a gram-negative bacillus was obtained. The muscle was reddish brown and without evidence of myocarditis. Microscopically there was no noteworthy change.

The kidneys appeared little altered excepting slight congestion. Microscopically, the glomeruli and tubules showed some evidence of degeneration in many of the animals. In a few, foci of hemorrhages were present. The liver parenchyma was in some instances slightly fatty. No areas of focal necrosis were found. The spleen was acutely swollen, without other noteworthy change.

I wish now to correlate certain features of the rabbit disease with those in the human influenzal lesions. This is done because of the general similarity of the two diseases in their symptomatology and epidemiology and possibly also in their etiology. With reference to etiology it is quite certain that *B. bipolaris* is the cause of the rabbit influenza. A similar organism, the Pfeiffer bacillus, is often associated with human influenza but presumably is only a common secondary invader and not the primary cause. It no doubt often plays a rôle in the causation of influenzal pneumonia and being somewhat similar to *B. bipolaris* it was thought a comparison especially of the pulmonary lesions in these two conditions would be of value.

A comparison of *B. bipolaris* and *B. influenzae* (Pfeiffer bacillus) has already been made by Davis.⁴ While similar in many ways certain distinguishing features exist, symbiosis and the hemophilic property being the most important; some other points of difference exist which need not be detailed here. It will be sufficient to state that these organisms cannot be considered identical or even very closely related.

Rabbit bronchopneumonia and human influenzal bronchopneumonia reveal somewhat similar gross alterations if approximately the same stages of the disease are taken. In the former, on opening the pleural cavities, the lungs do not completely collapse and the pleural cavities usually contain a moderate amount of fluid, seldom blood tinged and containing fibrin. The picture in human influenzal bronchopneumonia is an excessive amount of blood tinged fluid, usually remarkably free from fibrin. On the pleural surfaces in both diseases are seen frequently small petechial and confluent hemorrhages. In rabbits the distribution of the consolidated areas is variable; in the acute cases they are for the most part situated in small patches near the periphery of the lung; in the more chronic ones they tend to become confluent and may be located more centrally. In several specimens the centers of these consolidated areas appeared to be necrotic and were studded with

⁴ Jour. Infect. Dis., 1913, 12, p. 42.

little yellow pin point size foci which on scraping often yielded plugs of necrotic material. While human influenzal pneumonia presents in the main a similar pathological picture one obvious difference is the lack of the central necrosis in the small consolidated areas and a greater tendency to become confluent, resulting in the massive confluent pseudolobar pneumonia, so-called.

Microscopically the rabbit bronchopneumonia shows a more marked perivascular edema and leukocytic infiltration than does the human type. The central necrosis above mentioned is much in evidence in the rabbit lung while little if any mention is made of it in the human disease. The exudative material is perhaps richer in cell content than is the case in human influenzal bronchopneumonia. Fibrin is not especially abundant, simulating therefore the human influenzal bronchopneumonia. Small miliary abscesses are not infrequently found. Focal necrosis of the pulmonary blood vessels was not evident in the rabbits as described by LeCount⁵ in human influenzal bronchopneumonia.

In order to compare the pathogenesis of these two infections it will be necessary to discuss experimental pneumonia. There are two principal theories with respect to the initial mode of pneumonic infection, namely, the hematogenous which has received little experimental support, having consistently failed in the hands of Wadsworth,⁶ Rasquin⁷ and Armstrong,⁸ and the bronchiogenic, which has received a certain amount of confirmation in the experimental production of pneumonia in animals by various methods of intratracheal or intra-bronchial insufflation.

Müller⁹ undertook a study of the pathogenesis of aspiration pneumonia experimentally produced in rabbits by vagotomy. From his observations he inferred that the bacteria gained entrance into the pulmonary tissue at the point where the cuboidal epithelium of the terminal bronchiole gave place to the flattened epithelium of the alveolar duct and atrium and that the invasion was facilitated by the mechanical injury produced by aspirated foreign material. He established the fact that further spread of the infection was by way of the interstitial tissue of the lung framework and by way of the alveolar walls.

⁵ Jour. Amer. Med. Assn., 1919, 72, p. 1519.

⁶ Am. J. Med. Sc., 1904, 127, p. 851.

⁷ Arch. med. exper. et d'anat. path., 1910, 22, p. 804.

⁸ Brit. Med. J., 1914, 2, suppl. 57.

⁹ Arch. klin. Med., 1902, 74, p. 80.

Blake and Cecil¹⁰ state that pneumonia was consistently produced in normal monkeys by intratracheal injections of pneumococci and showed that the pneumonia produced ran a clinical course identical with that of man. They furthermore state that attempts to produce pneumonia by subcutaneous or intravenous inoculations have consistently failed and therefore conclude that pneumonia is a bronchiogenic and not a hematogenous affair.

They, however, obtained their experimental results by intratracheal inoculation of the animals through needle puncture. Winternitz, Smith and Robinson¹¹ have pointed out that in such inoculations, the needle, though sterile on entry, is unquestionably infected when it is withdrawn and consequently a possible path of infection to the lung may be found elsewhere than through the lumen of the trachea. They demonstrated that the submucosa of the trachea and bronchi furnishes a pathway of infection to the lung. It contains a rich plexus of lymphatics prominent everywhere, devoid of valves. There is a continuity throughout this lymphatic system so that bacteria which once find their way into it may easily spread.

In this rabbit bronchopneumonias produced by intratracheal injections through a soft rubber catheter, the process was apparently not bronchiogenic in character, for the patchy, confluent consolidations were not situated near the large bronchi or the hilum of the lung; the larger bronchi in the greater proportion of cases were uninvolved and no evidence of bacterial passage through the mucosa was discernible. The consolidations were situated indiscretely over the lung surface, both near the periphery and the center and often numbering as high as 15 to 20 to a lobe. Microscopically the lymphatics, especially the perivascular, were dilated and infiltrated with leukocytes. Bacteria were not seen in them. From this it seems reasonable to assume that the rabbit bronchopneumonia is a hematogenous or lymphogenous affair in contradistinction to human influenzal bronchopneumonia. This view is in harmony with the fact that this infection in rabbits often manifests itself as a septicemia, indeed is often called rabbit septicemia.

No direct evidence was obtainable as to the site of the primary invasion. That the bacteria passed through the mucosa somewhere near the hilum of the lung and entered the lymphatic system seems probable for two reasons: first, the catheter was inserted to the

¹⁰ J. Exp. Med., 1920, 31, p. 445.

¹¹ Bull. Johns Hopkins Hosp., 1920, 31, p. 63.

bifurcation of the trachea and second, the peribronchial lymphatics were involved in most instances.

In no instance have observations been recorded of the tendency to multiple abscess formation in human influenzal bronchopneumonia comparable to the striking process seen in rabbit pneumonia in which the consolidated areas undergo an intense central necrosis without delimitation of the process by capsule formation. Large regional softenings as seen at times in human lungs following pneumonia were not observed in the rabbits.

SUMMARY

Rabbit bronchopneumonia may be caused by *B. bipolaris* which belongs in the hemorrhagic septicemia group. The disease may be produced experimentally.

Under natural and experimental conditions a bronchopneumonia appears which is usually distributed throughout all portions of the lung, both peripherally and centrally, the microscopic picture being dependent on the stage of the disease. Peribronchial and perivascular infiltration of the lymph spaces and regions of central necrosis were the most constant lesions.

A comparison of rabbit pneumonia and influenzal pneumonia as described by various writers indicates certain points of similarity, but also certain differences. Grossly and microscopically both are bronchopneumonic processes, often confluent in type. They differ, however, in that in rabbit pneumonia the perivascular and peribronchial leukocytic infiltration of the lymph spaces and the regions of necrosis in the consolidated portions are more constant and striking features. The latter appears to be especially distinctive of rabbit pneumonia.



H. T. Seegarick

WILLIAM THOMPSON SEDGWICK, 1855-1921

Professor Sedgwick's name has appeared on the title page of *THE JOURNAL OF INFECTIOUS DISEASES* ever since the Journal was founded in 1904, and it is with deep regret that we here record his death on Jan. 25, 1921.

William Thompson Sedgwick was born of old New England stock at West Hartford, Conn., Dec. 29, 1855. When 26 years old he married Mary K. Rice of New Haven. He received degrees at Yale (S. B.), and at Johns Hopkins (Ph. D.), where he worked in the department of physiology with Prof. H. N. Martin. In 1883 he went to the Massachusetts Institute of Technology in Boston where he remained in active service as head of the Department of Biology and Public Health to the end of his life.

His principal books are: in coöperation with Professor E. B. Wilson, a "Text Book of General Biology" (1886), one of the first books to make clear to the beginning student the fundamental resemblances of all living things; "The Principles of Sanitary Science" (1902), an illuminating discussion of certain phases of public health work; *The Human Mechanism* (with Dr. Theodore Hough) and, in coöperation with Prof. H. W. Tyler, a "Short History of Science" (1917), the notable outcome of a life-long interest in the history of science and the biographies of scientific men. Besides these he published scores of articles and public addresses—in which he was a master—and participated largely in scientific administration and organization in the state of Massachusetts and throughout the country. His truly amazing activity and versatility were among his most striking characteristics. While recognizing clearly the danger of diffuseness in others, his own interest in different fields was so keen and his sense of public duty so compelling that he frequently found it difficult to deny himself to worthy causes outside his daily path. The influence that he exerted in this way in his community and the amount that he accomplished were far beyond the capacity of the ordinary person, and mark the measure of the man.

Few men in this country have had so great a share as Sedgwick in the development of bacteriology and public health. He was a pioneer in the introduction of bacteriologic ideas into the United States and in

WILLIAM THOMPSON SEDGWICK

their application to public health problems. Although not himself a laboratory worker he had the far rarer gift of inspiring in others a love for science and for scientific investigation. The number of pupils in his department was never large, averaging hardly three or four graduates a year, but few American teachers of his generation could point to so many pupils doing productive work in so many different fields. The present professor of sanitary engineering at Harvard, of public health at Yale, of protozoology at Columbia, of physiology at Cincinnati and at Virginia, and many others received their early training and their inspiration in Sedgwick's laboratory. In 1906, Sedgwick's pupils on the occasion of the twenty-fifth anniversary of his doctorate published a volume of "Biological Studies" written especially to do him honor. At the annual meetings of the American Public Health Association it has been the custom for a number of years for Sedgwick's graduates to gather in friendly conference and send him a telegram of greeting. It is perhaps significant that from no other single educational institution—had the attempt been made—could have been mustered so many public health workers, often numbering between thirty and forty, as acknowledged their loyalty to Sedgwick and sent their greetings to the "Chief."

There was more than professional fealty and admiration in the feeling of all of Sedgwick's pupils for their master. He was a friend to whom they could always appeal for advice and counsel, and was himself always watchful of their needs and desires. He followed his pupils' careers with the most careful attention and never hesitated to use his great influence to further their best interests. Always intolerant of intellectual slackness and never mistaking interest for ability, once his confidence was obtained he was untiring in his support. Sedgwick's unusual success in "placing" the graduates of his laboratory was largely due to the deep personal interest he took in each individual and to his shrewd judgment of capabilities and resources which such friendly relations made possible.

It is not as a student, not as an investigator, but as a great teacher, a great publicist, a potent shaper of the lives of young men and above all as a lover of his fellows, that Professor Sedgwick has impressed himself on his time and on hundreds of his contemporaries who will do the best they can to hand on the torch.

E. O. J.

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS

K. F. MEYER

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

The part played by the human carrier in the spread of typhoid fever is well known. The experience of various health departments shows that today flies, food (exclusive of milk), privies and sewage are relatively small factors in the dissemination of this disease. In the state of Massachusetts, according to O'Donnell,¹ few cases are due to water, especially in recent years, and in general no municipal supply is considered as a dangerous source of typhoid infection. Contact with true typhoid has been responsible for a few cases in homes and neighborhoods, while carriers have been proved to be responsible for 41.6% of cases of milkborne typhoid from 1915 to 1918, inclusive. It is therefore evident that carriers are a larger factor in the spread of typhoid than at present realized and are probably also the cause of an appreciable number of typhoid cases of unknown origin. This statement is supported by the fact that from 1915-1919, 14 carriers caused 249 cases of typhoid fever. Similar observations have been made by Chesley² and his associates, who traced 213 cases of typhoid fever in Minnesota to 30 carriers, or an average of 7 cases each. In California also the persistence of typhoid fever is definitely linked with carriers. Certain data collected in the army camps (Camp Dix) and in the Expeditionary forces clearly suggest that, in some instances, this mode of infection was responsible for small epidemics. Moreover, Garbat³ has recently estimated that 55% of all typhoid may be traced directly or indirectly to carriers. The general reduction of typhoid in the United States has enabled the health authorities to devote more attention to the prevention of contact and carrier cases. A statement by Meader⁴ summarizes the present situation in an excellent manner. He says: "By far the most difficult source of infection to trace and the most baffling to control is the typhoid carrier, and methods for the cure of typhoid carriers are among the most needy research problems."

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¹ *Am. Jour. Public Health*, 1920, 10, p. 517.

² *Jour. Am. Med. Assn.*, 1917, 68, p. 1882.

³ *Ibid.*, 1916, 67, p. 1493.

⁴ *Mod. Med.*, 1920, 2, p. 244.

An elimination of typhoid fever can obviously only be expected when the problem dealing with the prevention and cure of carriers has been solved. Förster⁵ has stated that if we can cure carriers we can get rid of typhoid fever. Unfortunately, it must be admitted that, hitherto, no certain method has been devised for rendering carriers typhoid-free. Biologic, as well as chemical, preparations have been applied to a limited number of intestinal carriers without any constant result. Surgical treatment, while not perfect, is at present considered the best available.

Since it is possible to produce in rabbits a condition similar to the carrier state in man, the new science of chemotherapy of bacterial infections has been tried extensively. The success so far achieved in rendering the rabbit carriers typhoid-free can be considered encouraging, but in no way striking. In all the experiments pertaining to the typhoid problem, it appears that therapeutic attempts have been made on a process, the pathogenesis of which is unknown, or in its best form very superficially investigated. If the numerous tests had been crowned with success, which, as already stated has not been the case, one could attribute the result more to chance than to a well planned systematic investigation. Therapeutic studies must be preceded by an experimental analysis of the factors leading to a persistence of the typhoid bacilli in certain tissues, and with it to the carrier state. It is unquestionably true that the prevention of typhoid carriers can only be accomplished when the pathogenesis of this condition is known in detail.

These and similar considerations suggested in 1915 a systematic study of the typhoid infection in various laboratory animals. No attempt was made in using the words of Metchnikoff and Besredka,⁶ "de résoudre le problème de la fièvre typhoïde si difficile et si compliqué." It was intended to place our knowledge concerning typhoid infection in rabbits on a broader basis. The subject was approached by making use of the well-known gall-bladder infections readily produced in these animals. It was soon realized that other laboratory animals must be included in the scope of the experimental observations. In dealing with so intricate a problem, on which a great deal had been written and which had been considered experimentally from so many different angles, the best working plan consisted in treating the typhoid bacillus and its allied organisms as living entities with an adaptive organization

⁵ München. med. Wchnschr., 1908, 55, p. 1.

⁶ Ann. de l'Inst. Pasteur., 1911, 25, p. 193.

and to follow them step by step in their passage through the animal body. The various phases of this investigation thus far completed with the aid of my collaborators will be summarized in a series of papers which will be submitted for publication as rapidly as other duties permit the undertaking.

The papers in preparation cover the following subjects: The Optimum H-ion Concentration for Growth of *B. typhosus* and *B. paratyphosus* A and B (paper 2); Do Carrier Strains differ from Strains Recently Isolated from Ordinary Typhoid Cases? (paper 3); A Comparative Study of the Infections Produced by Intravenous Injections of Typhoid, Paratyphoid A and B bacilli in Normal and Immunized Rabbits (paper 4); The Mechanism of Gallbladder Infection in Laboratory Animals (5); The Reaction and the Physiology of the Gallbladder and Hepatic Duct Bile of Laboratory Animals (6); The Bacteriostatic and Germicidal Properties of Bile (7); The Influence of the H-ion Concentration on the Growth of *B. typhosus* in Mediums Containing Bile or Bile Salts (8). Subsequent papers are planned to deal with: Feeding Experiments on Rabbits with *B. typhosus* and *B. paratyphosus*; Diet, Starvation and Immunization as Factors in the Production of the Intestinal Carrier State in the Rabbit; The Physical Properties of the Inoculum in the Production of Carriers; The Examination of Animal Stools as an Aid in the Diagnosis of the Carrier State; Renal Carriers in Rabbits; The Fate of *B. typhosus* and *B. paratyphosus* B Intravenously Inoculated into Guinea-Pigs, Cats, Rats and Monkeys; Typhoid and Paratyphoid A and B Infections Produced in Guinea-Pigs by Gallbladder Injections; Feedings of *B. typhosus* in Normal Guinea-Pigs and Those Suffering from "Deficiency" Disease; A Study of the Tissues Obtained from Experimental Carriers; and finally, A Correlation of the Experimental Results with the Facts Available from the Study of Human Carriers.

THE OPTIMUM HYDROGEN-ION CONCENTRATION FOR THE GROWTH OF *B. TYPHOSUS*, AND *B.* *PARATYPHOSUS* A AND B

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. II

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California Medical School, San Francisco*

It is known that *B. typhosus* and the allied organisms of this group grow best in a neutral or slightly acid medium. The present study was undertaken to determine the probable range of growth and the optimum H-ion concentration.

There are two methods commonly employed to measure the H-ion concentration of a solution. The first is by means of the H-electrode, the second is the colorimetric method. For very accurate work, in which slight changes in H-ion must be determined, or in which the greatest possible accuracy is to be attained, the first method of measuring H-ion concentration is generally used. However, when the changes in H-ion concentration extend over a broad range, and when the method of experiment calls for a large number of determinations of only relative accuracy, the second method is to be preferred. This is particularly true when the solutions to be tested are more or less complex and the salt and protein content interferes with the electrometric determinations.

The ability of certain solutions to resist changes in H-ion concentration is called its buffer action. The body fluids, ordinary Witte's peptone solution, solutions of weak acids, such as phosphates, borates and acetates, and many others, exhibit this property. This behavior of certain substances in solution has been used by several investigators in the preparation of solutions of known H-ion concentration. Sørensen¹ carefully studied such a set, as did Walpole² and Palitsch³; Clark and Lubs⁴ have recently modified these sets slightly. In our work, we have found it necessary to prepare these buffer solutions in a range extend-

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¹ Biochem. Ztschr., 1907, 7, p. 131.

² Biochem. Jour., 1910, 5, p. 207.

³ Biochem. Ztschr., 1912, 47, p. 1.

⁴ Jour. Bacteriol., 1917, 2, p. 1.

ing from $P_H + 4.0$ to $P_H + 9.0$. These solutions were carefully prepared⁵ and checked (through the kindness of Dr. Schmidt) with a H-electrode.

Up to recent years one of the main objections to the use of the colorimetric method for the determination of H-ion concentration in bacteriologic work was the scarcity of brilliant indicators covering the entire range of H-ion concentration. Phenolphthalein, an indicator most commonly used, has a range on the alkaline side from $P_H + 8.0$ to $P_H + 10.0$. Litmus is not sensitive or brilliant enough for colorimetric work. Methyl red has been used and has proved very satisfactory. It passes from its full acid color into its alkaline color as the H-ion concentration falls from $P_H + 4.8$ to $P_H + 6.4$. Through the work of Clark and Lubs,⁴ however, several new and brilliant indicators have become available for this work. Methyl red, bromthymol blue, cresol red and thymol blue were the indicators most generally used in this work.

There has been a great deal of discussion relative to the death point and its relation to the H + ion concentration. Bruenn,⁶ using mixtures of lactates as well as acetates of a definite H + ion concentration, came to the conclusion that the disinfection which takes place at high H + ion concentrations depends on the $P_H +$. He found that the death point of *B. coli* was between $P_H + 4.7$ and 4.4 ; for *B. typhosus* between $P_H + 5.6$ 5.0-4.7. We have not noted quite as large a range in our experiments, as will be indicated later. Winslow and Lockridge⁷ have shown that typhoid bacilli are a little less than half as resistant as *B. coli* to dilute acids. Using HCl and H_2SO_4 , the toxicity depends on the H + ion concentration; the undissociated part of the molecule exerts no toxic effect. Organic acids, such as benzoic or acetic acids, are toxic for these organisms in strengths in which they are only slightly dissociated. The toxicity is the result of the undissociated part of the molecule. The accumulation of similar organic acids might account for the phenomena observed by Clark, that the final H + ion concentration is lower in more highly buffered mediums.

In order to determine the degree of acidity or alkalinity necessary to inhibit *B. coli* in the animal body, Shohl and Janney⁸ studied the growth of these organisms in urine at varying H + ion concentrations. They

⁵ We are indebted to Mr. P. Edson for the preparation and standardization of these solutions.

⁶ Jour. Urology, 1917, 1, p. 211.

⁷ Jour. Infect. Dis., 1906, 3, p. 547.

⁸ Jour. Urology, 1917, 1, p. 211.

showed that these organisms were inhibited in their growth at a $P_H +$ of 4.6-5.0 on the acid side, and from $P_H +$ 9.2-9.6 on the alkaline side. The optimum growth for *B. coli* was between 6.0-7.0. They also tested the Rawlings strain of *B. typhosus* (our strain 6) and state that the typhoid bacillus has narrower limits, showing no growth at $P_H +$ 5.0 on the acid side, or $P_H +$ 8.8 on the alkaline side. Quite recently Winslow, Kligler and Rothberg⁹ found that *B. typhosus* (Rawlings' strain) produces, inside of 24-96 hours, in hexoses an acid reaction of $P_H +$ 5.5-5.0, which remains practically constant.

TABLE 1

HYDROGEN-ION CONCENTRATION OF MIXTURES OF SALT-FREE BROTH, HYDROCHLORIC ACID OR SODIUM HYDROXIDE AND PHOSPHATES

10 cc broth + 2 cc 5M phosphates + HCl or NaOH + H₂O = 13 cc

Tube No.	0.1 N NaOH, C c	0.1 N HCl, C c	0.5 N KH ₂ PO ₄ , C c	0.5 N Na ₂ HPO ₄ , C c	Hydrogen-ion Concentration of Mixture, P_H
		P_H of Medium = 7.4			
	3.0	9.0
1	2.4	2	8.8
2	2.4	2	8.6
3	2.0	...	0.04	1.96	8.4
4	1.3	...	0.1	1.9	8.2
5	1.0	...	0.12	1.88	8.0
6	0.6	...	0.14	1.86	7.8
7	0.2	1.8	7.6
8	0.4	1.6	7.4
9	0.7	1.3	7.2
10	...	0.5	0.8	1.2	7.0
11	...	0.6	1.0	1.0	6.8
12	...	0.8	1.2	0.8	6.6
13	...	1.1	1.45	0.55	6.4
14	...	1.6	1.7	0.3	6.0
		P_H of Medium = 6.4			
15	...	0.6	1.7	0.3	6.0
16	...	0.8	1.8	0.2	5.8
17	...	1.0	1.9	0.1	5.4
18	...	1.7	2	...	5.0
19	...	2.1	2	...	4.8
20	...	2.4	4.3

The exact effect of variations in the $H +$ ion concentration of culture mediums on *B. typhosus* has not been investigated extensively. It was thought advisable to investigate this problem before attempting other work in which variations in the $H +$ ion concentration are concerned. Moreover, it will be evident from the observations reported in subsequent papers (6, 7 and 8) of this series, that a detailed knowledge of the H -ion requirements of an organism is absolutely essential before an explanation can be attempted relative to the behavior of a bacterium in the body fluids or the secretions of an animal.

⁹ Jour. Bacteriol., 1919, 4, p. 471.

Technic.—Preparation of Mediums: The mediums were prepared by allowing 500 gm. of lean veal to infuse in 1000 cc of tap water on ice over night. This infusion was boiled for 20 minutes, strained through cheese cloth and allowed to cool. The fat was removed by filtration, 1% "Difco" peptone was added, and the medium adjusted to a reaction of $P_H + 7.4$. After stabilization in the Arnold for 30 minutes, the broth was filtered and sterilized by fractional sterilization in live steam for 3 consecutive days. This "salt free medium" was made in large quantities. The same lot of medium was used for the entire series of experiments.

TABLE 2
INFLUENCE OF SALT CONCENTRATION ON THE GROWTH OF B. TYPHOSUS
INCUBATION 18 HOURS AT 37 C.

P _H 7.0 Molal Concen- tration	Growth			
	NaCl		KCl	
	Kearney	Blair	Kearney	Blair
1.0	±	±	—	—
0.5	++	++	++	++
0.3	+++	+++	+++	+++
0.1	+++	+++	+++	+++
0.06	+++	+++	+++	+++
0.04	+++	+++	+++	+++
0.02	+++	+++	+++	+++
Salt free broth	+++	+++	+++	+++

TABLE 3
INFLUENCE OF PHOSPHATES ON B. TYPHOSUS BROTH AND PHOSPHATE MIXTURE
(7 PARTS Na₂HPO₄ + 3 PARTS KH₂PO₄)

+ 0.1 cc of 1:10,000 20-hour broth culture + distilled H₂O = 5 cc

Strain								
Kearney			Jacobs			Blair		
Tube Number	Molal Concen- tration of Phos- phate	Growth	Tube Number	Molal Concen- tration	Growth	Tube Number	Molal Concen- tration	Growth
20	0.4	±	25	0.4	±	30	0.4	±
21	0.3	++	26	0.3	++	31	0.3	++
22	0.2	+++	27	0.2	+++	32	0.2	+++
23	0.1	+++	28	0.1	+++	33	0.1	+++
24	...	+++	29	...	+++	34	...	+++
Salt-free broth			Salt-free broth			Salt-free broth		

To each 10 cc of medium NaOH or HCl was added in order to obtain the desired reaction. Two cc of 0.5M Na₂HPO₄ and 0.5M KH₂PO₄ mixtures (Na₂HPO₄ being alkaline and KH₂PO₄ being acid in reaction) were added in order to keep the H-ion concentration constant during the course of the experiment. By combining these solutions in certain proportions a series of tubes varying in reaction from $P_H + 4.3$ to $P_H + 9.0$ were obtained. Such a series is shown in table 1.

Three c c quantities were tubed and inoculated with 0.1 c c of a 1:10,000 dilution of a 24-hour "salt-free broth" culture. Growth was determined after 24 hours' incubation by the plate method. Triplicate plates were poured with veal infusion agar, $P_H + 6.8-7.2$, and counted after 24 hours. The remainder of the culture was sterilized in the Arnold for 30 minutes and the H-ion concentration determined. A control tube was also incubated and heated in the same way and the H-ion concentration determined. Variations in the H-ion concentrations were seldom found, except on the extreme alkaline side. These results are in accord with those obtained by Dernby and Avery¹⁰, in their work on the growth of pneumococcus.

Isolation of Strains.—The strains were isolated on brilliant green mediums and transferred to peptic digest agar slants. For the experiments the cultures were purified on brilliant green or plain agar plates. Single colonies were transferred to veal infusion agar slants ($P_H + 6.8-7.2$) and kept at room temperature.

TABLE 4
OPTIMUM GROWTH OF *B. TYPHOSUS*; STOCK CULTURES

Blair 2*			Jacobst†			Number 1‡			Kearney§		
P_H Cont.	P_H 24- Hour Cult- ure	Growth per C c	P_H Cont.	P_H 24- Hour Cult- ure	Growth per C c	P_H Cont.	P_H 24- Hour Cult- ure	Growth per C c	P_H Cont.	P_H 24- Hour Cult- ure	Growth per C c
4.3	4.3	2,000				4.6	4.5	2,000	4.3	4.3	
4.8	4.8	97,000	4.8	4.8	0	4.9	4.8	2,000	4.8	4.8	<10,000
5.0	5.0	41,000,000	5.1	5.1	21,600,000	6.6	6.4	423,000,000	5.2	5.2	92,500,000
6.4	6.4	480,000,000	6.6	6.4	383,000,000	6.6	6.6	645,000,000	5.4	5.4	185,000,000
6.6	6.6	580,000,000	6.8	6.6	503,000,000	6.8	6.8	606,000,000	5.6	5.6	225,000,000
6.8	6.8	876,000,000	7.0	6.8	480,000,000	7.0	7.0	650,000,000	6.0	6.0	960,000,000
7.0	7.0	400,000,000	7.4	7.0	260,000,000	7.2	7.2	525,000,000	6.6	6.4	1,500,000,000
7.4	7.4	236,000,000	8.3	7.9	216,000,000	7.4	7.4	482,000,000	6.8	6.8	1,500,000,000
8.4	8.4	143,000,000	8.9	8.9	<1,000,000	8.3	8.6	320,000,000	7.0	7.0	2,500,000,000
9.0	8.8	8,250				8.9	8.9	200,000	7.4	7.2	1,810,000,000
									7.6	7.4	1,400,000,000
									8.2	8.0	171,000,000
									8.4	8.3	3,000,000
									8.6	8.6	<100,000

* Isolated from the blood in a severe case of typhoid complicated with cholecystitis and gall stones.

† Isolated from blood of a fatal case of typhoid, November, 1917.

‡ Isolated from a renal carrier, April, 1917.

§ Typical typhoid isolated from blood of a moderately severe case of typhoid, July, 1916.

Experimental Data.—The Influence of Salts on the Growth of *B. typhosus*: Preliminary experiments were conducted to determine the effect of varying concentrations of KCl, NaCl, KH_2PO_4 , and Na_2HPO_4 on *B. typhosus*. The results of these experiments are shown in tables 2 and 3.

According to table 3 a 0.4 M solution of Na_2HPO_4 - KH_2PO_4 mixture, is bacteriostatic, although it does prevent growth; a 0.3 M solution of phosphate is, however, more inhibitive than a 0.3 M solution of NaCl or KCl, etc. Since Na_2HPO_4 and KH_2PO_4 dissociate into 3 ions as compared to the 2 ions of the NaCl or KCl solution, a molal solution

¹⁰ Jour. Exper. Med., 1918, 28, p. 345.

of Na_2HPO_4 or KH_2PO_4 has a greater osmotic pressure than a molal solution of KCl or NaCl . The ratio of the comparative strength of the solution to the osmotic pressure is about as follows: Molal concentration of NaCl : molal concentration of $\text{Na}_2\text{HPO}_4 = 2:3$ Isosmotic solutions of NaCl and Na_2HPO_4 , therefore, produce about the same effect on *B. typhosus*. The phosphate concentration used in these experiments was kept far below that concentration which causes inhibition.

OPTIMUM GROWTH OF *B. TYPHOSUS* AND *B. PARATYPHOSUS* A. AND B.
IN THE STANDARD "BUFFERED" MEDIUM

The determination of the optimum H-ion concentration for the growth of *B. typhosus* was conducted as previously stated. The results are shown in the following tables:

TABLE 5
OPTIMUM GROWTH OF *B. TYPHOSUS*; STOCK CULTURES

Strain No. 3*			Strain No. 5†			Strain No. 7‡		
P_H Cont.	P_H 24-Hour Cul- ture	Growth per C c	P_H Cont.	P_H 24-Hour Cul- ture	Growth per C c	P_H Cont.	P_H 24-Hour Cul- ture	Growth per C c
4.6	4.6	130	4.6	4.6	100	4.6	4.6	100
5.0	5.0	99,000	4.9	4.9	19,000	5.0	5.0	18,700
6.0	6.0	384,000,000	6.0	5.9	323,000,000	6.6	6.5	836,000,000
6.5	6.4	1,163,000,000	6.4	6.3	583,000,000	6.8	6.8	1,605,000,000
6.6	6.4	1,473,000,000	6.6	6.6	600,000,000	7.0	7.0	1,326,000,000
6.8	6.7	1,540,000,000	6.8	6.8	773,000,000	7.4	7.4	810,000,000
7.0	7.0	2,130,000,000	7.0	7.0	630,000,000	8.7	8.7	50,500,000
7.4	7.4	810,000,000	7.4	7.4	570,000,000			
7.8	7.8	530,000,000	8.6	8.1	353,000,000			
8.7	8.7	50,500,000						

* Isolated from urine of patient suffering from renal calculi, November, 1916.

† Isolated from blood on the twelfth day of the disease, in a moderately severe case of typhoid, June, 1916.

‡ Isolated from blood in a moderately severe case of typhoid, October, 1916.

It will be seen from tables 4 to 7, inclusive, that the range of growth of *B. typhosus* is large. The optimum growth is between $P_H + 6.8-7.0$, or slightly on the acid side. Slight variations may occur, but most of the strains tested showed the same optimum range of growth. *B. coli* (Shohl and Janney), as compared to *B. typhosus*, shows a more pronounced optimum zone of growth and a large range. The results of 17 experiments on stock cultures have been averaged and are shown in the chart by the unbroken line. The logarithms of the number of organisms per cubic centimeter of medium are plotted as ordinates against the H-ion concentrations as abscissae.

TABLE 6

OPTIMUM GROWTH OF B. TYPHOSUS; RECENTLY ISOLATED CULTURES

Eldridge*				Lung Strain†				M. Strain§				Joe Strain¶				"C" Strain			
P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.
5.8	5.8	150,000,000	6.0	6.0	300,000,000	5.8	5.8	5.9	5.9	190,000,000	5.6	5.6	6.0	6.0	254,000,000	6.0	5.9	280,000,000	6.0
6.4	6.4	250,000,000	6.4	6.4	350,000,000	6.4	6.4	6.4	6.4	940,000,000	6.0	6.0	6.0	6.0	526,000,000	6.2	6.0	270,000,000	6.2
6.6	6.6	310,000,000	6.6	6.6	465,000,000	6.6	6.6	6.6	6.6	1,120,000,000	6.4	6.4	6.4	6.4	690,000,000	6.6	6.6	449,000,000	6.6
6.8	6.8	460,000,000	6.8	6.8	480,000,000	6.8	6.8	6.8	6.8	1,010,000,000	6.8	6.8	6.8	6.8	726,000,000	6.8	6.8	410,000,000	6.8
7.0	7.0	430,000,000	7.0	7.0	405,000,000	7.0	7.0	7.0	7.0	960,000,000	7.0	7.0	7.0	7.0	886,000,000	7.0	7.0	392,000,000	7.0
7.2	7.2	410,000,000	7.2	7.2	445,000,000	7.2	7.2	7.2	7.2	1,010,000,000	7.2	7.2	7.2	7.2	856,000,000	7.6	7.4	456,000,000	7.6
7.7	7.7	350,000,000	7.4	7.4	440,000,000	7.6	7.6	7.6	7.6	710,000,000	7.4	7.4	7.4	7.4	826,000,000	8.0	7.9	386,000,000	8.0
8.6	8.1	260,000,000	7.6	7.6	425,000,000	7.6	7.6	7.6	7.6	370,000,000	7.8	7.8	7.8	7.8	640,000,000	8.6	8.5	239,000,000	8.6

* Eldridge isolated from blood of a moderately severe case of typhoid, February, 1919.

† Eldridge isolated from stool of same patient. Optimum H-ion concentration determined immediately after isolation.

‡ Isolated from a case of influenza followed by a moderately severe case of typhoid. B. typhosus isolated from sputum.

§ Isolated from blood in a moderately severe case of typhoid, February, 1919.

¶ Isolated from blood in a mild case of typhoid in a vaccinated person, February, 1919.

TABLE 7

EFFECT OF VARYING H-ION CONCENTRATIONS ON GROWTH OF PARATYPHOSUS A AND B

Para A II* Original Culture				Para A III† Bile Strain				Para A II‡ Duodenal Strain				Para A §§				Para B ¶¶			
P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.	P _H 24-Hour Cul-ture	Growth per C e	P _H Cont.
4.3	4.3	100	4.3	4.3	33,500	4.3	4.3	4.3	4.3	300	6.0	6.0	6.0	6.0	708,000,000	6.0	6.2	740,000,000	6.0
4.8	4.8	2,000	5.2	5.1	28,200,000	4.8	4.8	4.8	4.8	2,500,000	6.4	6.4	6.4	6.4	746,000,000	6.4	6.4	910,000,000	6.4
5.0	5.0	16,300,000	6.4	6.4	636,000,000	5.2	5.1	5.2	5.1	11,000,000	6.6	6.6	6.6	6.6	801,000,000	6.6	6.6	960,000,000	6.6
6.6	6.6	460,000,000	6.6	6.5	1,003,000,000	6.4	6.4	6.4	6.4	425,000,000	6.8	6.8	6.8	6.8	1,266,000,000	6.8	6.8	1,190,000,000	6.8
6.8	6.8	513,000,000	6.8	6.8	553,000,000	6.8	6.8	6.8	6.8	543,000,000	7.0	7.0	7.0	7.0	1,136,000,000	7.0	7.0	1,410,000,000	7.0
7.0	7.0	683,000,000	7.2	7.1	600,000,000	7.2	7.0	7.2	7.0	543,000,000	7.2	7.2	7.2	7.2	796,000,000	7.2	7.2	980,000,000	7.2
7.5	7.5	708,000,000	7.5	7.4	626,000,000	7.5	7.4	7.5	7.4	536,000,000	7.4	7.4	7.4	7.4	896,000,000	7.6	7.6	990,000,000	7.6
7.8	7.8	556,000,000	8.6	8.2	386,000,000	8.6	8.2	8.4	8.0	216,000,000	7.8	7.8	7.8	7.8	336,000,000	7.8	7.8	830,000,000	7.8
8.3	8.2	450,000,000	8.8	8.6	433,000,000	8.8	8.6	8.8	8.6	184,000,000									
8.8	8.6	105,600,000																	
8.9	8.9	>50,000,000#																	

* Received from Captain Nichols. Isolated from Private Fraser in Colorna, Dublin, Mexico, August, 1916.

† Same strain taken from gallbladder carrier rabbit 816 days after infection.

‡ Same strain taken from duodenum of same rabbit.

§ Received from Captain Nichols, March, 1916, isolated from a blood culture.

¶ Received from the department of bacteriology of the University of Bern, December, 1913.

Colonies too numerous to count.

It is evident that the rise in the curve on the acid side is greater than on the alkaline side. In other words, even though *B. typhosus* grows best in a slightly acid environment, it may be slightly more tolerant to alkalis than to acids.

One interesting point brought out by these experiments was that slight changes, near the limiting H-ion concentration, produced more marked changes in growth than approximately the same changes near

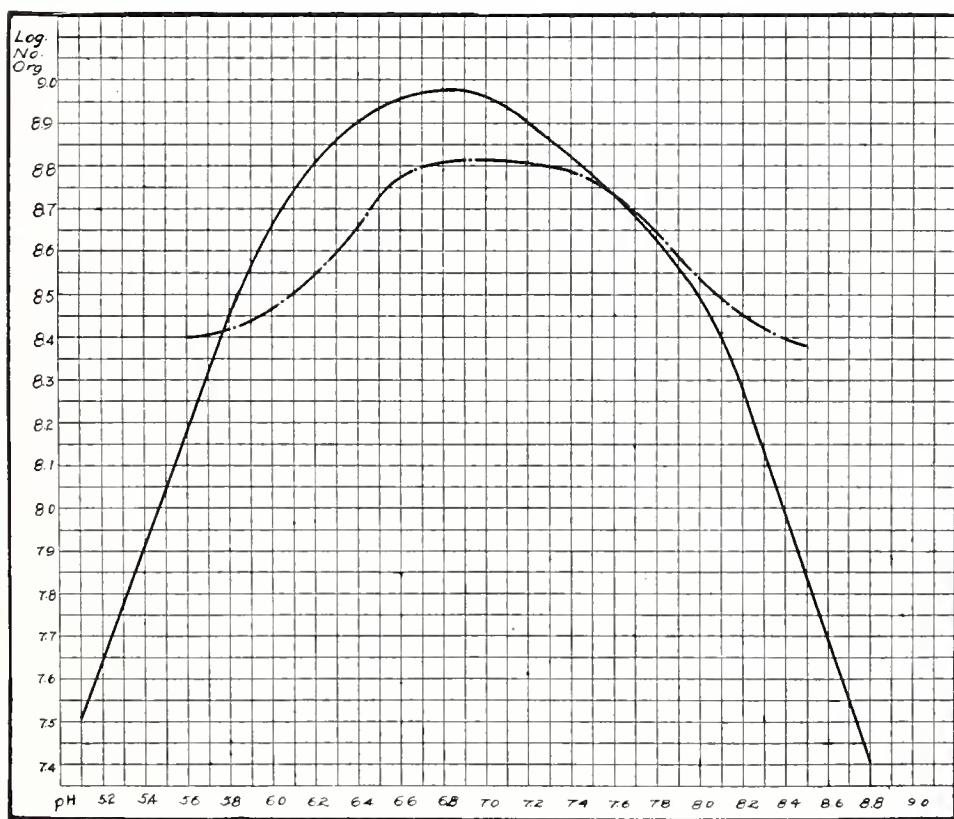


Chart 1.—Growth curve of *B. typhosus*. The growth of stock strains is represented by the unbroken line; the growth of recently isolated strains, by the dash and dot line.

the optimum H-ion concentration. For instance, in the "Blair" strain (table 4), a change from $P_H + 4.8$ -5.0 caused a change in growth from 97,000 organisms per c c to 41,000,000 organisms per c c; while a change from $P_H + 6.4$ - $P_H + 6.6$ resulted in a change of 10,000,000 organisms from 480,000,000-580,000,000. The "Kearney" strain failed to grow at at $P_H + 4.3$ and at 4.8, while at $P_H + 5.2$, 92,500,000 organisms were

counted per c.c. A change from 6.4 to 6.8 produced no recordable change in growth.

Another important observation was made. Recently isolated cultures had a decided optimal zone of growth which was more marked than in the stock cultures. The dash and dot line in the chart represents the results obtained by averaging 6 experiments conducted with recently isolated strains. Stock cultures exhibited an optimum zone with a definite optimum point of growth, while recently isolated strains developed in an optimum zone. Moreover, the range of growth of the latter strains was greater and the tolerance for alkali was more marked. This was particularly striking with the "C" strain, table 6. The growth of this strain was as profuse at $P_H + 7.9$ as at $P_H + 7.0$. The inhibition at $P_H + 8.6$ was not as marked as in the stock cultures. These observations were suggestive of bacterial adaptation to alkali and acid, and were considered of utmost importance in connection with the problem of urinary and gallbladder carriers.

These findings have some bearing on the selection of the medium to be employed in the isolation of *B. typhosus*. The optimum reaction for Endo-medium is $P_H + 7.8$ - $P_H + 8.4$. Such a concentration of alkali is inhibitive for *B. typhosus*. The optimum reaction for a brilliant green medium is $P_H + 7.0$. As far as reaction is concerned, the latter medium is therefore preferable to Endo's agar. It was pointed out by Stickel and Meyer¹ that a negative finding of a stool containing few typhoid bacilli may be misleading when the usual Endo-medium is used. The results obtained in the present study fully confirm these views.

Table 7 shows the effect of varying H-ion concentrations on the growth of paratyphosus A and paratyphosus B.

It is obvious from table 7 that the paratyphoid strains showed an optimum growth at the same H-ion concentration as the typhoid strains, but exhibited a marked plateau on either side of the optimum. The para B strains investigated are more alkali-tolerant than the para A strains.

Para A 2 was isolated from a rabbit which was sacrificed on the 816th day as a chronic gallbladder carrier. The bile and the duodenal strain, being recently isolated, were expected to grow in a plateau curve, but in our tests the reverse was noted, namely, the bile strain showed a decided optimum at $P_H + 6.5$ while the duodenal strain grew in a

¹¹ Jour. Infect. Dis., 1913, 23, p. 48.

plateau from $P_H + 6.6$ to $P_H + 7.4$. The original stock culture behaved in a similar manner, namely, a plateau from $P_H + 6.6$ - $P_H + 7.5$. In fact, the growth at $P_H + 7.5$ was slightly greater than at $P_H + 6.8$. These differences, which were determined by repeated tests, cannot be explained. It is not unlikely that the prolonged sojourn in a blocked gallbladder, the exposure to various products of inflammation, produced a tolerance to acid. This observation supports the conception that bacteria may undergo a process of adaptation while resident in the tissues of an immune host. Changes in the growth curve may be one of the newly acquired properties of the protoplasm which can be readily recorded. Growth curve studies may therefore be of value in the study of bacterial adaptation.

SUMMARY AND CONCLUSIONS

B. typhosus has a range of growth from $P_H + 5.0$ to $P_H + 8.6$ with an optimum growth at $P_H + 6.8$ - $P_H + 7.0$ in a salt-free veal infusion broth. Above or below these limits the resulting growth in comparison is very slight.

Large variations in the H-ion concentration near the optimum zone produce only slight effects on the growth of the organisms, while slight variations at the limiting zone produce a marked effect. These observations are fully in accord with the results reported by Cohen and Clark¹² in their studies on the growth of certain intestinal organisms at different concentrations. In the region near the optimum H-ion concentration the tolerance for alkali seems to be slightly greater than for acid.

Stock cultures isolated from stools, blood and urine of typhoid patients or carriers have a more decided optimum than recently isolated cultures of similar cases. In such cultures the plateau of the growth curve is much more pronounced and extends over a wider range than in stock cultures. The latter is suggestive of microbic adaptation to changes in H-ion concentration in body fluids, particularly urine and bile.

B. paratyphosus A and B have a range of growth at varying H-ion concentrations similar to that of *B. typhosus* but exhibit a greater tolerance for alkali than *B. typhosus*.

¹² Jour. Bacteriol., 1919, 4, p. 409.

DO "CARRIER" STRAINS DIFFER FROM STRAINS ISOLATED FROM ORDINARY TYPHOID CASES ?

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. III

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This question involves the consideration of several factors which may play a part in the successful production of a high percentage of experimental gallbladder or renal carriers. It should be emphasized that this study was primarily undertaken to elucidate certain facts recorded in the literature pertaining to animal carriers. It was not the intention to attempt to prove or to refute the conception that the typhoid strains isolated from human carriers may differ in their invasive or infective properties, a phase of the problem which is practically impossible when one considers that the experimental reproduction of typhoid fever in laboratory animals cannot be accomplished. The customary method of testing the virulence of typhoid strains by intraperitoneal injections into guinea-pigs does not reproduce the conditions as they operate in human infections and the data collected by this method cannot be used for comparison. Neither Lentz¹ nor Ledingham² noted by the use of this procedure differences between the virulence of the carrier strains and those isolated from ordinary typhoid cases. Some incomplete observations of Ledingham, however, suggest that the virulence of the typhoid strains isolated from one and the same carrier may vary for guinea-pigs at different times of the year. For example, a strain isolated from an intestinal carrier exhibited a reduced virulence during the winter months. Using a similar method of testing for virulence, Remlinger³ noted that typhoid bacilli found in liver abscesses were avirulent, and Niepratschk⁴ isolated from a renal carrier a strain of low virulence. Furthermore, Levy and Gaehtgens⁵ recorded by means of bacteriotropin tests the observations

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* Dr. C. R. Christiansen, who died from influenza in October, 1917, conducted during the years 1915-16 the majority of the experiments to be reported. This paper should stand as a memorial to his enthusiastic and resourceful collaboration in our typhoid studies.

¹ Klin. Jahrb., 1905, 14, p. 475.

² The Carrier Problem in Infectious Diseases, London, 1912, p. 110.

³ Compt. rend. Soc. de biol., 1897, 4, p. 110.

⁴ Ztschr. f. Hyg. u. Infektionskr., 1909, 64, p. 454.

⁵ Ergebn. d. allg. Path. u. path. Anat., 1915, 18, p. 491.

that the virulence of the typhoid bacilli excreted by typhoid patients was as a rule greater than the one of carrier strains. It has been suggested by Hilgermann⁶ that the relative infrequency of infections in the entourage of carriers may be due to variations in the virulence of the organism and that this diminishes with the continued parasitic existence of the latter in the organs of its host. Based on some observations on rabbits Wagner and Emmerich⁷ came to the same conclusions. The well-known studies by Metchnikoff and Besredka⁸ on experimental typhoid fever demonstrated that typhoid bacilli shed by a chronic carrier are pathogenic for small laboratory animals, but that they are unable to transmit typhoid to chimpanzees. The general notion that carrier strains are less virulent than those isolated from acute typhoid cases finds, therefore, some justification, and the question of the occurrence of saprophytic and parasitic strains is again placed in the foreground. Closely interwoven with the answer to the original question is the consideration of the possibility that carrier strains may possess specific elective properties for the tissues from which they are isolated. Ledingham,⁹ in discussing this subject, expresses the opinion that this hypothesis can be proved. A carrier strain that has sojourned for long periods in man may conceivably be more highly endowed with those properties which permit of its continued vegetation within the body after the infection is past than a typhoid strain responsible for a water-borne epidemic, which gets little opportunity of becoming accommodated to the human organism. As far as we know, the conception of elective organotropism in the sense of Rosenow¹⁰ has not been considered experimentally for the typhoid bacillus but has been fully proved by Fränkel and Much¹¹ for a paratyphoid B bacillus obtained from a case of purulent cholecystitis.

Early in the course of our typhoid work, we felt the necessity of studying these problems pertaining to the inherent properties of the typhoid bacillus before we undertook a systematic study of the factors responsible for the occurrence of gallbladder, liver or renal carriers. We were fortunate in having access to carrier strains, which were responsible for extensive and serious epidemics. The dynamic properties of some of these strains as far as they concern man had been well

⁶ *Klin. Jahrb.*, 1908, 19, p. 463.

⁷ *Med. Klin.*, 1916, 12, p. 819.

⁸ *Ann. d. l'Inst. Pasteur*, 1911, 25, p. 193.

⁹ *The Carrier Problem in Infectious Diseases*, pp. 75 and 76.

¹⁰ *Jour. Infect. Dis.*, 1916, 14, p. 527.

¹¹ *Ztschr. f. Hyg. u. Infektionskrankh.*, 1911, 69, p. 342.

investigated epidemiologically. These investigations were at our disposal through the publications and verbal informations obtained from Drs. W. A. Sawyer and F. G. Cummings. Additional cultures were isolated from carrier cases in the wards of the University of California Hospital and compared with recently isolated strains of our own and those we received through the courtesy of Drs. H. T. Chickering, J. V. Cooke, and the Bureau of Communicable Diseases of the California State Board of Health. The available carrier strains were not very numerous, but could at least be considered representative of the variety of typhoid bacilli as they occurred in a large community.

The analysis of these typhoid strains covered originally two questions: First, are there any cultural or biochemical differences between carrier strains and those recently isolated from acute typhoid cases? Second, do carrier strains exhibit a specific elective behavior for certain tissues in the rabbit, and can this elective localization be acquired by successive passage through the gallbladder of this species of animal? A study of carrier strains isolated at different time intervals from one and the same case was contemplated, but the specimens necessary for this investigation could not be procured with desirable regularity.

In the course of the analysis of the preliminary data, it was realized that an additional question could be advantageously investigated on the same animals, namely, Do immunized rabbits develop a gallbladder carrier state more readily than normal animals? This question was first raised by Fornet¹² in connection with his theory that the carrier state is to be regarded as a consequence of established partial immunity to the typhoid bacillus, and that, in fact, the bacilli discharged by the carrier may not necessarily be descendants of those that caused the primary infection. A reinfection takes place, which, according to his views, is not accompanied by any clinical symptoms. The region attacked by the bacilli in this reinvasion would have acquired, in consequence of the primary infection, such a tolerance to the micro-organisms as to render its saprophytic existence possible. Fornet mentioned, further, in his first article that animal experiments to support his contention were in progress. According to a more recent review,¹³ he apparently gained from a few preliminary experiments the impression that immunized rabbits developed more readily and more regularly a typhoid carrier state by alimentary infection than nonimmunized

¹² Ztschr. f. Hyg. u. Infektionskrankh., 1909, 64, p. 365.

¹³ Ergebn. d. innern Med. u. Kinderheilk., 1913, 11, p. 205.

animals. In the same article,¹⁴ he suggested that similar results may be obtained in animals infected by the intravenous route. We will have occasion to discuss, in a paper dealing with the feeding of typhoid and paratyphoid bacilli to rabbits, the impossibility of producing a gallbladder infection by the alimentary method. This phase of Fornet's theory is unquestionably incorrect. One may recall in this connection that the carrier state supposed to have been produced by the inoculation of one half of a standard 10% blood-agar culture of a certain strain of *B. typhosus*, has been recommended as a means for testing the immunizing power of a given typhoid vaccine. Nichols¹⁵ and others were unable to repeat these results. The experiments of the latter are supported by our own observations, namely, that typhoid immunized rabbits develop a carrier state in a higher percentage of instances than normal animals. The occurrence of local infections in immunized or possibly sensitized animals has some bearing on the general problem of focal infections in man. Numerous observations reported in the literature indicate that immunization does not prevent the development of local infections in rabbits. One gains the impression that such procedures quite frequently favor it. In this connection the work of Faber¹⁶ on streptococcic arthritis, and of Wadsworth¹⁷ on pneumonia in rabbits, should be mentioned. Furthermore, Creig¹⁸ reports the development of a gallbladder carrier state in rabbits progressively immunized with living cholera-like vibrios, while Flu¹⁹ notes similar lesions in animals repeatedly infected with Flexner dysentery bacilli. The factors responsible for these results have been investigated, and will be discussed in this and in subsequent papers.

THE CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF "CARRIER" STRAINS

During the last five years 14 *B. typhosus* strains, isolated from carriers in California, were studied in detail by means of the usual cultural and carbohydrate tests. The histories of the strains used in the animal experiments are indicated in table 1. The epidemics caused by strains 10 and 21, are discussed by Sawyer²⁰ and the infections

¹⁴ Ibid., p. 216.

¹⁵ Jour. Exper. Med., 1914, 20, p. 573.

¹⁶ Jour. Exper. Med., 1915, 22, p. 615.

¹⁷ Am. Jour. Med. Sc., 1904, 128, p. 851.

¹⁸ Indian Jour. Med. Research, 1915-16, 3, pp. 259 and 397.

¹⁹ Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.

²⁰ Jour. Am. Med. Assn., 1912, 58, p. 1336; 1914, 63, p. 1537.

provoked by strain 4 have been reported by Cummings.²¹ Strains 18, 49, 1 and 84 are stock strains, or were isolated by us from necropsy material, or from secretions of carriers under our control. These cultures, in addition to 7 others, were studied by the methods recently described, in connection with the analysis of 2 irregular typhoid strains.²² No differences from the ordinary typhoid strains could be noted; the behavior on carbohydrate medium, milk and on rhamnose plates was typical. All the strains were rapid xylose fermenters and failed to attack dulcitol in liquid mediums. These observations are quite in harmony with the findings of E. Müller,²³ who studied 19 carrier strains. According to his results, the strains of *B. typhosus* obtained from intestinal carriers showed no abnormalities, nor did the cultures exhibit any indication of newly acquired properties.

All strains were specifically agglutinated by a polyvalent typhoid immune serum. The variation of the individual strains differed in no respect from those commonly encountered by Bull and Pritchett²⁴ with the ordinary typhoid strains. As most of our tests were made previous to the findings of Hooker,²⁵ who clearly demonstrated consistent antigenic differences among some strains of *B. typhosus*, absorption tests were not done with the freshly isolated strains, and our inquiry along these lines is therefore incomplete. Recently a few tests conducted with the strains cultivated on agar for from 2-7 years showed that strains 10, 4 and 84 belonged to group 2 and strains 18 to group 3 of Hooker's classification, while strains 21, 49 and 1 exhibit the characteristics of group 1. Hooker had already found, in his studies in 1916, that our strain 18 and his strain 4 fell in group 3. It is not unlikely that such tests would have produced different results had they been carried out when the strains were originally isolated. The existence of atypical and irregular typhoid strains in carriers should be seriously considered in the light of our recent observations. Moreover, the publication of Downs²⁶ suggests the presence of antigenic differences among typhoid strains and a subdivision into at least 5 groups. Confirmation of these observations as to the occurrence of various types of typhoid bacilli would be of considerable epidemiologic importance in the analysis of "carrier" borne typhoid outbreaks, and may perhaps

²¹ Jour. Am. Med. Assn., 1917, 68, p. 1163.

²² Meyer, K. F., and Neilson, N. M.: Jour. Infect. Dis., 1920, 27, p. 46.

²³ Centralbl. f. Bakteriol. I. 1909, 53, p. 209.

²⁴ Jour. Exper. Med., 1916, 24, p. 35.

²⁵ Jour. Immunol., 1917, 2, p. 1.

²⁶ Abstr. Bacteriol., 1920, 4, No. 56, p. 19.

explain the variable infectivity of carriers. Gruber,²⁷ for example, was able to trace a carrier through the peculiar behavior in glycerol of the causative typhoid strain, the so-called *B. metatyphosus* of Mandelbaum. An intensive study of carrier strains would perhaps also explain the findings of Schlemmer,²⁸ who noted that one and the same carrier may eliminate at varying time intervals strains of typhoid bacilli, which are nonsusceptible to the bactericidal properties of the same immune serums. These observations involuntarily lead to a consideration of the virulence of our carrier strains. There is no experimental animal on which one can successfully test the relative virulence of typhoid strains, but to satisfy our curiosity we applied the method of Ledingham²⁹ to strains 1, 49 and 84. These organisms killed guinea-pigs weighing 250 gm. in quantities of not less than 1 c c of a 24-hour old broth culture. Strain 49 killed in 2.5 c c amounts. In comparison with the ordinary typhoid strains 40, 41, 54, 55 and 56, which were fatal to guinea-pigs in 1-2 c c amounts, the virulence of the 3 carrier strains cannot be considered exceptionally low.

Our findings on carrier strains differ therefore in one respect from those reported by Lentz, Ledingham, E. Müller and others, and justify the definite and final conclusion that culturally and biochemically carrier strains do not differ in any way from those recently isolated.

THE BEHAVIOR IN THE RABBIT OF CARRIER STRAINS AND OF STRAINS RECENTLY ISOLATED FROM ACUTE INFECTIONS

In accordance with the problems discussed in the introduction, we tested the theory of Fornet and investigated the conception of elective organotropism advanced by Rosenow and Brown,³⁰ using a number of carrier strains at our disposal. These tests were paralleled by similar experiments with recently isolated strains and with those artificially cultivated for several years. Such a comparison was necessary in order to prove the following points: (a) the validity of the conclusions drawn from the experiments conducted with carrier strains; (b) the observations of Weinfurter,³¹ Nichols,¹⁵ and others which indicated that freshly isolated strains produced gallbladder lesions in a higher

²⁷ Arch. f. Hyg., 1913, 80, p. 272.

²⁸ Ztschr. f. Immunitätsforsch. u. exper. Therapie, 1911, 9, p. 149.

²⁹ The Carrier Problem in Infectious Diseases, pp. 109 and 110.

³⁰ Arch. Int. Med., 1919, 23, p. 185.

³¹ Centralbl. f. Bakteriöl., I. O., 1915, 75, p. 379.

percentage of instances than old strains; and (c) the assumption that a "certain" strain of *B. typhosus* cultivated on blood agar is necessary to provoke a carrier state in rabbits. Furthermore, our ulterior motive in conducting these tests centered on the desire to find a typhoid strain which was particularly suited for carrier studies on rabbits.

Methods.—The rabbits used in this series of experiments weighed between 2,000 and 3,000 gm., and were supplied from the same source. During the years 1915, 1916, and 1917 these animals were free from coccidiosis and spontaneous cholecystitis. Little attention has been paid to this infection by the majority of workers on experimental typhoid, and sweeping conclusions on gallbladder infections have been drawn from the results obtained on rabbits, which possessed, according to the published protocols, livers heavily infested with coccidiosis. Some investigators (Gotschlich³²) are aware of the fact that coccidiosis of the liver favors the localization of bacteria in the biliary passages and the gallbladder, while others intentionally or unintentionally ignore this infection. Experimental conclusions on cholecystitis appear justified only when one is certain that the animal does not possess predisposing infections. Many statements relative to the value of a method for the production of carriers, expressed in percentages of infections, are seriously invalidated by the utter neglect of these fundamental requirements.

The animals were kept on a mixed oat, hay and cabbage diet, in single "Lewis" cages and in a fly proof isolation room. The sawdust bedding was disinfected by lysol or strong lye solutions.

Immunization was accomplished by at least 6 intravenous inoculations, at 6-7 day intervals, of heat killed (53-54 C.) (McCoy), tricrosolized, autogenous cultures grown on rabbit-blood agar. The vaccine amounts were increased progressively from 1/20 to 1/2 of a slant. Ten to 15 days after the last injection of the vaccine, the animals were infected with the same strain of typhoid bacilli in a living state. For these tests the cultures were purified on Endo plates, and kept on veal agar or grown on rabbit-blood agar slants for at least 5 generations before use. The preparation of the mediums, the selection of the tubes and proper slanting were conducted in accordance with the specifications ordinarily employed. We intend to discuss in another paper of this series the observations made in connection with the cultivation of typhoid strains on rabbit-blood agar, but attention is now called to the fact that in our experience never more than 40-60,000 million typhoid bacilli grew on the specified agar surface. The figures determined by Gay and Claypole and faithfully copied by Stone,³⁸ namely, 1,400,000 million organisms, are probably incorrect. In order to reduce the initial mortality caused by the inoculation of large doses of living typhoid bacilli into rabbits, we determined when possible the minimum lethal dose, and found that from 2-5 billions per kilogram of rabbit weight are fairly well borne by normal animals weighing over 2,000 gm. Expressed in terms pertaining to blood-agar slants, these amounts varied from 1/10 to 1/4 slant per rabbit. All inoculations were made with unstrained suspensions in sterile saline solution using a marginal vein, and in one series of animals the renal artery.

³² Handb. d. path. Mikroorganism, Ed. 2, 1912, 1, p. 217.

³⁸ Jour. Infect. Dis., 1919, 25, p. 290.

The rabbits were killed in from 20-35 days after the inoculation. Cultures were made on Endo-agar plates or by enrichment of portions of the organs in 10% ox bile rabbit broth. The major portion of the data presented was collected early in the course of our study on experimental typhoid carriers. The conception of the carrier state at this period was comparatively simple, namely, the presence of typhoid bacilli in the bile and gallbladder wall constituted a positive result.

The data presented in tables 1 and 2 should be considered from this point of view. An animal was noted as "died" when it succumbed to the intoxication in the first 24-48 hours.

TABLE 1

GALLBLADDER CARRIERS PRODUCED IN RABBITS BY THE INJECTION OF OLD AND RECENT "CARRIER" STRAINS OF B.TYPHOSUS

Strain	Age of Strain	No. of Animals		Positive		Negative	
		Immune	Normal	Immune	Normal	Immune	Normal
Stool and Osteomyelitis:							
10 "O" responsible for 26 contact cases in 3½ years	3½ years	5	5	1	1	4	4 (died)
18 "Cr" tibial abscess 2 years after an attack of typhoid fever	3 years	4	2	1	0	3	2
21 "L" responsible for 93 cases in one epidemic	1 month	6	8	5	5	1	3 (died)
4 "H" responsible for 23 cases in a food epidemic	2 months	3	—	3	—	—	—
49 gallstone, negative, no history of typhoid fever	3 days	3	3	0	1	3	2
Urine:							
"1" renal carrier, injection made through the left renal artery	3-4 months	6	2	6	2	0	0*
84 "renal carrier," right typhoid pyelonephritis	Immediately urine sediment of 200 cc of urine 10 billion organisms	2	2	1	2	1	0*
7 strains.....	Immediately to 3½ years	29	22	17 56%	11 50%	12 44%	19 50% 9% died

* Kidneys of all animals negative.

Experimental Data.—The various experiments are shown in concrete form in tables 1 and 2. It is quite apparent that with the exception of strains 21 and 1, none of the carrier strains possessed elective properties for the gallbladder of rabbits. The percentage of infections in 51 rabbits inoculated with carrier strains was lower than that found in 70 rabbits similarly infected with recently isolated strains of B. typhosus. The highest percentage of infections was noted with a freshly isolated strain 21 which was introduced intravenously, and with a 3-4 month old culture (1) which was injected through the left renal artery. The latter experiment will be discussed elsewhere more in detail, but in connection with it attention should be called to the fact

that operative technic, fasting of the animals with the resulting stasis in the gallbladder, unquestionably contributed to the permanency of the infection in the biliary passages. Furthermore, this experiment with strain 1, as well as that conducted with 84, lend little support to the contention that the parasitic typhoid strains isolated from chronic renal carriers possess for the rabbit at least elective organotropic properties.

TABLE 2

GALLBLADDER OR LIVER CARRIERS PRODUCED IN RABBITS BY THE INJECTION OF RECENT AND OLD STRAINS OF *B. TYPHOSUS* ISOLATED FROM STOOL BLOOD CULTURES

Strain	Age of Strain	No. of Animals		Positive		Negative	
		Immune	Normal	Immune	Normal	Immune	Normal
Recent Blood Cultures:							
24.....	28 days	10	12	8	5	2	7
26.....	55 days	2	2	1	2 (died)	1	0
27.....	6 months	1	1	1	1	0	0
28.....	5 months	1	2	0	0	1	2
29.....	5 months	2	2	1	1 (died)	1	1
36.....	53 days	1	2	0	1	1	1 (died)
37.....	2 months	1	1	1	0	0	1
38.....	1½ months	1	1	0	0	1	1
40.....	2½ months	1	1	0	1	1	0
41 (severe case).....	3 months	1	1	1	1 (died)	0	0
45.....	3½ months	1	2	0	1 (died)	1	1
46.....	2 months	1	1	1	0	0	1 (died)
48.....	1 month	2	1	2	1	0	0
50.....	1 month	1	1	1	1	0	0
52*.....	2½ months	2	2	0	1	2	1 (died)
53*.....	2 months	1	1	1	1 (died)	0	0
54*.....	2 months	1	1	1	1	0	0
55*.....	1 month	1	1	1	1	0	0
56*.....	1 month	1	1	1	0	0	1
Chr. 2.....	4 months	1	1	1	1	0	0
Total.....		33	37	22	20	11	17
Average percentage of carriers.....				66%	54.3% (25% died)	33%	45% (17% died)
Old Stool Cultures:							
15 ("I").....	3 years	2	0	1	0	1	0
17 (old).....	>3½ years	1	—	0	—	1	—
14 (Hopkins).....	>4 years	2	2	0	0	2	2
6 (Rawlings).....	15 years	9	5	2	0	7	5
23 (Dorset).....	16 years	2	2	1	1	1	1
Total.....		16	9	4	1	12	8
Average percentage of carriers.....				25%	11%	75%	88.8%

Quite recently Lentz, Hailer and Wolf³⁴ reported similar tests with eight carrier strains. Each culture was inoculated in one loopful amounts into 5 rabbits. Only 30% of the animals developed a carrier

³⁴ Arb. a. d. Reichs Gendhsamte, 1918, 51, p. 1.

state. These tests were undertaken to prove whether or not the strains employed by Doerr³⁵ and by Johnston³⁶ possessed specific elective properties, which could explain the high percentage of infected gall-bladders recorded by these writers. It is evident that our experiments confirming those of Lentz³⁴ fail to support the conception of elective parasitism.

Our observations support the findings of Weinfurter³¹ and of Nichols,¹⁵ namely, that recently isolated parasitic strains produce a higher percentage of carriers than old saprophytic strains. But it is also demonstrated that even such strains failed to produce biliary infections regularly. The high initiated mortality of 17-25% among the nonimmunized rabbits must, however, be considered a serious and expensive disadvantage.

How do our results compare with those reported by 15 other workers? We have attempted to analyze their reports and have summarized the data in table 3. In this summary it is difficult to estimate the number of organisms inoculated, the weight and age of the rabbits, and the time period elapsing between infection and necropsy, facts which interfere seriously in estimating the true value of the experiments. Making allowance for these and other factors like coccidiosis, individual resistance, diet, etc., it is quite obvious that the average percentage of gallbladder carriers produced does not exceed 65%. This figure may be accepted for our analysis. A variety of strains was employed by the different experimenters. Old and recently isolated strains and even carrier strains were chosen by Morgan,³⁷ by Hailer and Rimpau,³⁸ by Hailer and Ungermann,³⁹ and by Lentz, Hailer and Wolf.³⁴ The evidence supports our conclusions that recently isolated strains, whether of carrier or acute typhoidal origin, produce gallbladder infections more frequently than old stock cultures. Carrier strains offer no advantage over recently isolated strains of acute typhoid cases. In our opinion the recognition of the fact that a large number of organisms is necessary to insure biliary infection is important. In this connection the high percentage of deaths should also be considered. Most of the workers in this field of experimental pathology fail to state the actual loss of animals in the 24 to 72 hours

³⁵ *Centralbl. f. Bakteriol. I. O.*, 1905, 39, p. 624.

³⁶ *Jour. Med. Research*, 1917, 37, p. 189.

³⁷ *Jour. Hygiene*, 1911, 11, p. 202.

³⁸ *Deutsch. med. Wchnschr.*, 1912, 38, p. 2267.

³⁹ *Arb. a. d. Gsndhsamte*, 1914, 47, p. 451.

following the injection of large doses of living typhoid bacilli. In our series we recorded as high as 25% mortality in nonimmunized animals, and we intend to publish several series of tests in which the mortality rose to 50%. Every worker will agree that such a method is prohibitive and wasteful. It will be the purpose of subsequent papers to discuss the procedures that were found of value in overcoming this loss. By these methods it was possible to raise the percentage of positive carriers to from 90-100% of the animals infected.

TABLE 3
PERCENTAGE OF GALLBLADDER CARRIERS PRODUCED BY VARIOUS WORKERS

Author	Weight of Rabbits	Number of Bacteria Injected		Percentage of Carriers
		As Stated by Author	Estimated by Us	
Forster and Kayser.....	2.0 - 2.6	0.5-0.6 mg.	600-15,000 million	Not regular infected on 5th day
Doerr.....	1.13- 2.0	$\frac{1}{4}$ -2 loopful	600- 5,000 million	90% in 10 rabbits
Lemière and Abrami....	—	2 c c of Vidal's agglutination fluid	1,000- 2,000 million?	Irregular after 6th day, recovery
Chiarolanza.....	2.0 - 2.5	$\frac{1}{2}$ -4 loopful	1,250-10,000 million	74% in 23 rabbits
Blumenthal, E.	—	1 loopful	2,500 million per kg. = 5-9,000 million per animal	100% in 5 rabbits
Bully.....	1.16- 2.0	1 loopful per kg.	2,500 million per kg. 2,500-5,000 per animal	48% in 40 control rabbits
Morgan.....	Aver. 2.52	4 c c of broth	2,800-3,200 million	50% in 10 rabbits
Johnston.....	0.98-1.94	0.5 c c of an agar suspension	?	?
Perussia.....	—	1-4 loopful per kg.	2,500-10,000 million per kg.	46% carriers in 15 rabbits
Weinfurter.....	2.5	$\frac{1}{4}$ -3 loopful	600-7,500 million	53% in 58 rabbits
Gay and Claypole.....	2.0-2.5	$\frac{1}{2}$ standard blood agar slant 720,000 million (?)	20,000-25,000 million	93.0% in 28 rabbits 90.6% in 43 rabbits 74-76% in ? rabbits (Beckwith)
Cummins and Cumming	—	1/10 slant	2,500-4,000 million	71% in 7 normal and immune rabbits had liver infected; 29% in 7 rabbits had infected gallbladder
Nichols....	2.0-3.0	$\frac{1}{2}$ fatal dose = 1/10 slant of blood agar	4-5,000 million	21% in 16 rabbits, 28.8% in 45 rabbits, 40% in 10 immune rabbits
Hailer and Rimpau and Hailer and Ungermann	2.5-3.0	1 loopful per kg.	6,000-7,000 million per animal	75% in 61 rabbits killed 8-34 after injection
Lentz, Hailer and Wolf..	—	1 loopful	2-5,000 million	30% in 40 rabbits
Teague and McWilliams	1.4-2.4	Large dose 8,000-15,000 million	—	60% positive in 10 rabbits 3-24 days
Stone.....	1.7-4.3	$\frac{1}{2}$ blood-agar slant 460,000 million (?)	10-20,000 billion	92% (Beckwith states 94%) in ?
Beckwith.....	—	$\frac{1}{2}$ blood-agar slant	10-20,000 billion	100% in ? rabbits

Analyzing the data in tables 1 and 2, we found to our surprise that immunized rabbits became carriers more readily than nonimmunized animals, an observation which confirmed the theory of Fornet, to which

we had had no access at the time our deductions were originally drawn. Nichols¹⁵ also noted a higher percentage of infection among his immunized rabbits, as did Cummins and Cumming.⁴⁰ Certain requirements must be fulfilled to obtain these results. It was found that immunized rabbits eliminated a larger number of typhoid bacilli than normal animals when the test inoculation was made on the 6-12th day after the last injection of the vaccine. Additional factors unquestionably play an important rôle, but it is not the purpose of this paper to discuss them. So much can, however, be said: The evidence at our disposal does not permit us to conclude that the results obtained on immunized rabbits are applicable to man and to the human carrier state.

Furthermore, it is evident that the development of the carrier state in rabbits cannot be used as a test for the potency of vaccines. Our rabbits were protected by 6 to 7 inoculations of a fresh vaccine prepared from the strain which was used for the test inoculations. Moreover, the intravenous doses were not larger than those usually employed. It has been argued that Nichols obtained his gallbladder infections in immunized rabbits because the intravenous test dose of one half fatal dose = 1/10 slant = 4-5 billion organisms was sufficient to overcome the increased bactericidal effect of the blood (which, by the way, does not exist). If this argument is sound, the same conclusion must apply to those studies, in which about 720 billion (?) living *B. typhosus* were employed and in which the reverse is reported as having occurred. In this connection one may state that even immunization with living bacteria does not alter the final outcome. Independent of the degree of protection, a certain percentage of rabbits that have recovered will develop the carrier state. This result is not surprising when one appreciates that, according to Parker and Franke,⁴¹ whose studies we have confirmed by extensive experimentation, the destruction of intravenously inoculated typhoid bacilli progresses with the same speed in immunized as in normal rabbits. No difference can be recorded in the bactericidal power of normal and immunized rabbit tissues. The infection of the gallbladder and the persistence in it of viable bacteria depend on certain factors operative in the liver, its vascular system, the bile and the diet of the animal (coccidiosis excluded) and not on the bactericidal properties of the blood.

⁴⁰ Jour. Roy. Army Med. Corps, Lond., 1914, 22, p. 378.

⁴¹ Jour. Med. Research, 1919, 39, p. 301.

CAN TYPHOID STRAINS ACQUIRE ELECTIVE CHOLECYSTOTROPIC
PROPERTIES BY SUCCESSIVE PASSAGE THROUGH
THE GALLBLADDER?

The studies reported in this chapter were undertaken in 1915-16, when the influence of Rosenow's doctrines permeated the bacteriologic and medical literature. Elective localization had been induced with the plague bacillus, the staphylococcus, the streptococcus, the colon bacillus, the paratyphoid bacillus and perhaps the representatives of the *Brucella* group, but no reports on the typhoid bacillus were available. As we were searching in our carrier studies for an organism which regularly localized in the gallbladder, the contemplation and execution of a series of passage experiments had at that time some justification and interest. Today, after having analyzed the mechanism of this infection in the rabbit, our efforts of this period appear superfluous. The course of the experiment is shown in tabulated form in table 4. By direct inoculation into the gallbladder the recently isolated typhoid strain was perpetuated through 4 rabbits. Each successive infection was done by transference of the infected bile from the viscus of the preceding, laparotomized rabbit. The strain was lost in the third passage; 15 days after the inoculation into Rabbit 722 the gallbladder bile was found sterile. This observation proves in the first place the contention of Hailer and Ungermann³⁹ that even the method of intravesicular inoculation of typhoid bacilli is not absolutely dependable, and that persistence of the bacteria may be of comparatively short duration. Furthermore, this typhoid strain, after it had been resident in the gallbladder for 116 days, had not acquired any selective property for growth in this viscus. This conclusion is also supported by the tests conducted on 33 rabbits, which received intravenously the first or second generation of the bile culture on blood-agar slants. The percentage of carriers (45 and 22%) differed in no respect from that noted for the recently isolated, nonlapinized strains. One may, therefore, conclude that elective cholecystotropic properties cannot be conferred on typhoid strains by successive passage through rabbits. This conclusion has, during the past 4 years, been repeatedly confirmed by experiments with typhoid or paratyphoid strains, which had been resident in the gallbladder of the rabbit from 230 to 816 days. In not one instance did these carrier strains exhibit elective properties. Prolonged sojourn in the rabbit tissues produced changes in agglutinability, as has been observed by Lange and Roos⁴² and by Wagner

⁴² Arb. a. d. Kais. Gsndhtsamte, 1915, 50, p. 57.

and Emmerich.⁷ Nearly 50% of the gallbladder strains were temporarily inagglutinable. A paratyphoid A strain, which persisted for 816 days in the gallbladder and the liver, was hyperagglutinable and clumped spontaneously. Intravenous injections of 10,000 million bacteria of this strain (second generation) were well tolerated by 5 rabbits. At necropsy 10 to 25 days after the injection these animals exhibited normal gallbladders. The strain had undergone some fundamental changes, but had not acquired specific invasive properties. An explanation of the carrier state can therefore not be expected on the basis of an elective localization in the sense of Rosenow.

TABLE 4

PASSAGE OF A RECENTLY ISOLATED STRAIN OF *B. TYPHOSUS* (K) THROUGH THE GALLBLADDER OF SEVERAL RABBITS

1st Passage. Rabbit 790 (2d generation intravesicularly)	
14 days	
2d Passage. Rabbit 797 (bile of 790 intravesicularly) 35 and 70 days, resp.	Rabbit 723 intravenously 1st generation, positive
	Rabbit 724 intravenously 1st generation, positive
	Rabbit 725 intravenously 1st generation, negative
	Rabbit 726 intravenously 1st generation, negative
	Rabbit 791 intravenously 2d generation, positive (died)
	Rabbit 792 intravenously 2d generation, positive
	Rabbit 793 intravenously 2d generation, negative (died)
	Rabbit 794 intravenously 2d generation, negative
	Rabbit 795 intravenously 2d generation, negative (died)
	Rabbit 796 intravenously 2d generation, negative
	Rabbit 798 intravenously 2d generation, positive
3d Passage. Rabbit 722 (bile of 797 intravesicularly) Negative on 25th day	5th generation of 790 and 2d generation of 797
	8 immune rabbits, all negative
	14 normal rabbits, 5 died in 24 hours, positive
	2 positive on necropsy 7 negative on necropsy

SUMMARY

A comparative study of 14 carrier strains failed to reveal any striking differences between these strains and those isolated from acute typhoid cases. Seven carrier strains tested on rabbits by means of intravenous injections failed to exhibit specific elective cholecysto- and renotropic properties. Furthermore, it was impossible to confer such characteristics to a recently isolated strain of *B. typhosus* by successive passage through the gallbladder of rabbits.

It was, however, demonstrated that immunized rabbits inoculated with large doses of living typhoid bacilli exhibited gallbladder infections in a somewhat higher percentage of instances than normal rabbits. The theory of Fornet and the publications of other workers on experimental typhoid carriers are discussed and compared with our own observations.

A COMPARATIVE STUDY OF THE INFECTIONS
PRODUCED BY INTRAVENOUS INJECTIONS
OF TYPHOID, PARATYPHOID A AND
B BACILLI IN NORMAL AND
IMMUNIZED RABBITS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. IV

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In order to establish a "carrier state" with absolute regularity, it is unquestionably necessary to obtain as clear a picture as possible of the cycle of events which follows an intravenous injection of gram-negative bacilli in the rabbit. The results of a number of experiments, dealing with the fate and disappearance of the typhoid and paratyphoid bacilli traced from the blood through the various organs are presented in this paper. In subsequent publications of this series, the removal of typhoid bacilli from the blood, their appearance in the bile and urine, the behavior of the leukocytes, etc., will be treated separately. Observations recorded in paper 3 suggested an analysis of the distribution and disappearance of typhoid bacilli in the organs of immunized rabbits. This study was part of a more general analysis of the mechanism of typhoid immunity in the rabbit, guinea-pig, cat, rat and monkey, and was undertaken in the form of a survey to prove or to refute the rather plausible conception of a cellular immunity in typhoid fever.

Observations on guinea-pigs also suggested some experiments with paratyphoid A and B bacilli. It will be demonstrated that paratyphoid A behave like typhoid bacilli in normal and immunized rabbits, while the paratyphoid B bacilli are more rapidly destroyed in the immune than in the normal rabbit. The mechanism responsible for the resistance and the ability of the normal, immune or immunized rabbit to destroy a large number of bacteria of the typhoid-paratyphoid group has been

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investigated in a few preliminary experiments. But interwoven with all these detailed studies are the main questions: Why does the gall-bladder become infected? Why does this viscus quite frequently retain the infection? The experiments recorded in this paper contribute some facts to the solution of this problem.

The technical execution of the contemplated extensive experimental program progressed rather slowly on account of the war, but was practically completed, when the studies of Parker and Franke¹ and of Stone² appeared in print. The results reported by the latter are contrary to those reported by Parker and Franke; namely, Stone claims to have demonstrated that typhoid bacilli disappear "more quickly from the organs of immune than from normal animals"; while Parker and Franke find "no difference in the bactericidal powers of normal and immune rabbits to intravenous injections of typhoid bacilli." The observations of the latter workers are in accordance with those reported by Bail³ and our own, although the data presented in their paper, as will be discussed later, are open to serious criticism. On the other hand, we see no justification in the assumption of Stone that the results of Parker and Franke are probably due to the fact "that too many organisms were injected." A careful study and calculation of the figures published shows that Stone injected (with one or two exceptions) one third of a standard blood-agar slant or approximately 460,000 million (?), while Parker and Franke used on the average 39,627,000 (p. 306) to 530-616 million (p. 305) typhoid bacilli. This newly created controversy was investigated and was satisfactorily solved, confirming the findings of Parker and Franke, by a series of additional tests recorded in this paper.

Methods.—The rabbits used in the experiments recorded were all free from coccidiosis; a series in which one animal was found to be infested with these protozoa has been discarded for reasons repeatedly stated in previous papers. With a few exceptions, which are noted, one set of experiments was always conducted on animals of one and the same litter, or a combination of two litters. The latter procedure was necessary only when tests on immunized and normal rabbits were extended over an observation period of 72-96 hours. In order to exclude spontaneous infections with coccidia the litters were raised from does, which were known to be healthy, under our own supervision. This likewise excluded to a certain degree the well-known variable resistance to typhoid infections of rabbits of different breeds. Animals of definite age, weight, and of known ancestry were tested with varying amounts of living bacteria. It

¹ Jour. Med. Research, 1919, 39, p. 301.

² Jour. Infect. Dis., 1919, 25, p. 284.

³ Arch. f. Hyg., 1905, 52, p. 272.

appears appropriate to emphasize that experimental bacteriologic studies in which the resistance of the animals plays an important rôle should only be conducted on offsprings with a well-known ancestral history. This fact should govern future experimental work on animals. Some incomplete data at our disposal suggestively indicate that increased resistance to bacterial infections may be inherited; on several occasions it was noted that the offspring of intensively immunized mothers were destroying typhoid bacilli in a shorter time interval than the young of a nonprotected mother. Attention is also called to the family predisposition for urinary infections (Bell and Hartzell⁴).

The typhoid cultures employed were recently isolated and were either grown on peptic-digest agar or 10% rabbit blood-agar slants. The 18-24 hour old growth was suspended in salt solution strained through cotton, and the number of bacteria per c.c. of suspension was determined by the dilution plate method. All cultures of one series were made in peptic-digest agar of the same lot. This inexpensive medium surpasses the ordinary veal infusion or meat extract-peptone agar. It is most unwise to express the dosage in slants; uneven growth, as a result of repeated transplanting, etc., produces enormous errors.

In a few experiments a single strain and in others a mixture of 4 to 6 strains was employed. Observations to be reported elsewhere suggested the use of polyhomogenous bacterial antigens. It is selfexplanatory that the immunized rabbits were protected against all the strains included in the test cultures chosen for the infection.

The rabbits were inoculated slowly by various methods specified in the experiments. After varying intervals they were anesthetized with ether, and completely exsanguinated from one or both carotid arteries. The excised organs in toto were placed in Petri dishes and weighed. Pieces of the organs (varying from 0.5 to 6.0 gm.) were then removed, weighed on sterile paper, transferred to sterile mortars and pulped thoroughly with sand. The ground tissues were emulsified in sterile salt solution in the proportion of 1 c.c. to 100 mg. of tissue. Not infrequently the whole spleen, gallbladder wall, suprarenal, etc., were treated in this manner. The gallbladder wall was carefully dissected from the liver tissue and thoroughly rinsed in salt solution, which was removed by sterile filter paper. The suspensions were prepared without delay after the organs had been excised, and the emulsions were plated in peptic-digest agar in amounts representing from 0.1 to 0.000001 gm. of the organ. Saline was used for the dilution of the emulsion, and by proper shaking it was attempted to obtain a uniform suspension, and a breaking up of the agglutinated bacteria. The plates were counted after 36-47 hours' incubation at 37 C. All figures given in the tables are calculated for 0.1 gm. or 100 mg. of tissue; this number represents not infrequently the average of duplicate or triplicate observations.

Contamination of the plates occurred only in exceptional instances, unless the tissues had been secondarily invaded *intra vitam* by various types of bacteria. *B. bronchisepticus* in the lung cultures of rabbits was the only organism which required the use of slide agglutination tests for identification, and it invariably necessitated a repetition of the experiment. Streptococci in the gallbladder or kidney were readily differentiated. All experiments recorded have been performed in duplicate or triplicate, but only one experiment of each series illustrating the result will be given in detail in the tables of this paper.

⁴ Jour. Infect. Dis., 1919, 24, p. 628.

THE LOCALIZATION AND SUBSEQUENT DISAPPEARANCE OF *B. TYPHOSUS*
FROM THE ORGANS OF NORMAL RABBITS INOCULATED
BY WAY OF THE BLOOD STREAM

The studies of Wyssokowitsch,⁵ Bail,³ Arima,⁶ Bull,⁷ and Parker and Franke¹ dealing with the course of a typhoid bacillus infection in rabbits have established the fact that the inoculated bacteria accumulate most abundantly in the liver, next in the spleen and bonemarrow. Destruction of the micro-organisms apparently takes place in the blood of the general circulation, through the action of the leukocytes, or the endothelial cells which line the capillaries most numerous in organs where the bloodstream is slower (Wyssokowitsch). In the majority of instances the foregoing tests were made on rabbits inoculated by the intravenous route and with comparatively small numbers of typhoid bacilli. Moreover, the period of observation was rarely extended over 24 hours, and little or no attention was paid to the gallbladder and its content. It has been pointed out in the preceding paper, and by Nichols⁸ that large doses are necessary to ensure gallbladder infections, and that as a rule the injection of bacteria into the portal system produces a higher percentage of persistent carriers than the usual intravenous injection. The purpose of the experiments reported is to verify these claims.

Exper. 1. Relation of the Number of Typhoid Bacteria Injected to the Number of Bacilli Found in the Tissues and Bile Ten Minutes After Inoculations.—Three rabbits of a litter 4½ months old were injected under ether anesthesia with varying numbers of a polyhomogenous mixture of 4 recently isolated strains of *B. typhosus* (K, B1, B and I). The exsanguination of the animals began at the end of 10 minutes and was completed in 5-6 minutes. The organs were immediately excised and plated. The results are presented in table 1.

Study of the table clearly shows that the intravenous injection of only a large dose of typhoid bacilli leads to an elimination of organisms in the gallbladder or the hepatic duct bile, and that the distribution of the inoculated bacilli in all 3 animals is strikingly uniform. Even after complete exsanguination, the liver, the spleen, the bonemarrow, the lungs, the kidneys, and the gallbladder wall, in the order mentioned, retained the inoculated typhoid bacilli. Two observations are of impor-

⁵ Ztschr. f. Hyg., 1886, 1, p. 3.

⁶ Arch. f. Hyg., 1911, 73, p. 265.

⁷ Jour. Exper. Med., 1914, 20, p. 237; 1915, 22, pp. 475 and 487; 1916, 23, p. 419; 24, p. 25.

⁸ Jour. Exper. Med., 1916, 24, p. 502.

tance: First, the total number of bacteria recovered as estimated from the blood and tissue cultures was far below the actual number injected. This fact, originally observed by Bull, definitely indicates that inside of 20 minutes in vivo, and inside of 30-50 minutes in vitro—time which must be allowed for the necropsy and the preparation of the cultures—30-50% of the inoculated viable typhoid bacilli are sufficiently injured by phagocytosis or otherwise, or perhaps so completely clumped, that our plating method fails to record the true number present. For the

TABLE 1
DISTRIBUTION OF B. TYPHOSUS IN THE RABBIT FOLLOWING INTRAVENOUS INJECTION
OF VARYING AMOUNTS OF ORGANISMS
Litter: Injected and Exsanguinated Under Ether.

Tissue	Injection of 1 Slant = 38,500,000,000 Organisms	Injection of 1/10 Slant = 3,850,000,000 Organisms	Injection of 1/100 Slant = 385,000,000 Organisms
	Rabbit 1378 (W. 1775)	Rabbit 1377 (W. 1775)	Rabbit 1380 (W. 1575)
Left and central liver lobe.....	22,100,000	1,750,000	130,000
Right and central liver lobe.....	16,800,000	1,350,000	136,000
Gallbladder wall.....	130,000	18,800	2,100
Bile.....	75 (0.8 c c)	14 (2.0 c c)	0 (1.5 c c)
Spleen.....	7,600,000	4,020,000	142,000
Bonemarrow.....	2,700,000	530,000	52,000
Mesenteric lymph nodes.....	27,000	1,900	25
Lungs.....	750,000	48,000	>2,000 (few bronchi septicus col.)
Kidneys.....	156,000	9,800	300
Urine.....	47 per c c	0	0
Carotis blood.....	22, and 14,000,000 per c c		62, and 98,000 per c c
	18,000,000	1,310,000 per c c	80,000 per c c
Heart blood.....	11,000,000 per c c		32,000
Duodenal content.....	125 B. typhosus colonies	Sterile; negative for B. typhosus	Sterile
Sacculus rotundatus...			Neg. for B. typhosus
Appendix wall.....	500 B. typhosus colonies	150 B. typhosus colonies	1 B. typhosus colony

present it is impossible to state which one of the various factors is responsible, but it should be kept in mind that the endothelial cells can be phagocytically active even for a few hours after the death of the animal. Second, the percentage of bacteria accumulating in the liver depends somewhat on the total number of typhoid bacilli inoculated. For example, in rabbit 1378, which received a whole slant, the liver retained 12,870 million bacteria, or 34% of the total number injected; while rabbit 1377, which received 1/10 slant, registered only 859 million, or 22%; and rabbit 1380 (1/100 slant) showed 6.8 million, or 18% of the total. On the other hand, the spleen registered only 0.5% of the actual number injected. The liver, as a whole, however, retained

more than 50% of the number of bacteria demonstrated in all the other organs, blood, and skeletal muscles, etc., combined.

It should be pointed out that the distribution of the retained bacteria was uneven in the various portions of the liver, which was probably the result of an uneven dispersion of bacilli in the blood-stream. Furthermore, the gallbladder as an appendix of the liver receives an equal share of the bacteria accumulated in the liver. This statement is supported by the following figures estimated from a number of counts obtained in this and similar experiments:

Rabbit 1.—0.33 gm. of gallbladder wall retain 23.000 *B. typhosus*.
 0.33 gm. of liver retain 18.150 *B. typhosus*.
 Rabbit 2.—0.21 gm. of gallbladder wall retain 19.740 *B. typhosus*.
 0.21 gm. of liver retain 31.000 *B. typhosus*.
 Rabbit 3.—0.27 gm. of gallbladder wall retain 50.760 *B. typhosus*.
 0.27 gm. of liver retain 65.000 *B. typhosus*.

An intravenous injection of typhoid bacilli into the rabbit will therefore always lead to an invasion of the gallbladder wall. If a sufficiently large dose is given, the clumped bacteria in the capillaries of the wall will probably not remain innocuous, but may lead to changes which favor a chronic infection of the bile and the biliary system. This conception will be discussed in the course of the analysis of additional experiments.

In the kidneys only a small number of bacteria accumulated. It is, however, shown that the injection of a very large dose of typhoid bacilli leads to an elimination of these bacteria by the urine. This phase of the experiment will be taken up later.

This and similar experiments also demonstrated that a very large number of typhoid bacilli cannot be removed readily from the blood-stream inside of 20 minutes. Smears prepared from the heart blood of such animals revealed a few loosely accumulated bacilli, while preparations made from rabbits injected with a small number of typhoid bacilli usually showed no bacteria, or a few clumps of aggregated bacilli. It is, therefore, obvious that the usual mechanism of removal of typhoid bacilli introduced into the circulation of rabbits by clumping, and subsequent phagocytosis in the organs, so ably investigated by Bull,⁹ is considerably impaired, when overwhelming doses are used. The phenomenon of "agglutination" in platelets is probably closely connected with the mixing or dispersion of the heavy bacterial suspension with the

⁹ Jour. Exper. Med., 1915, 22, pp. 475 and 487.

blood, and the irregular gathering of the organisms in the lung capillaries; but this entire mechanism will be considered elsewhere more in detail. The inability of the choked organs to dispose of the retained typhoid bacilli constitutes an important factor in the formation of foci, which continue to maintain the bacteria in the blood stream.

These experiments also prove that a fairly large dose of over 1,000 million organisms is necessary to produce in the rabbit an elimination of bacteria in the bile after an ear vein injection. It is a debatable question whether the appearance of typhoid bacilli in the hepatic or cystic bile of rabbits, subsequent to an intravenous injection, can really serve as an indication that such an animal will become a chronic carrier or develop a cholecystitis. It is, however, certain that the body does not free itself by excreting the living bacteria in the bile and urine. The number of organisms so eliminated is, in proportion to the inoculum, very small; in our case, one five-millionth of the infecting dose of bacilli. Assuming for the present that the elimination of bacteria with the biliary secretion alone is responsible for a persistent gall-bladder infection, it is not surprising to record that Nichols⁸ Tanabe and Takeuchi¹⁰ and others noted a higher percentage of carriers in rabbits infected by way of the mesenteric than the ear vein. In their tests more organisms appeared in the bile after portal vein injections than after ear vein injections. We have compared the two methods of infection in several experiments, but have been unable to confirm these claims, as is shown by the results reported in the following:

Exper. 2. The Fate of Typhoid Bacilli Introduced Into the Circulation by Way of the Portal Vein and by Way of an Ear Vein. Comparison Between Intravenous and Subcutaneous Mode of Infection.—Two rabbits (1343 and 1342) of the same litter, 211 days old, received injections, using the ear vein, with 5,000 million typhoid bacilli strain K., and one rabbit (1349) received the same amount subcutaneously. Two rabbits (1344 and 1345), also of the same litter, were laparotomized under ether anesthesia and the bacilli were introduced into the circulation by way of a mesenteric vein. The animals were exsanguinated and their organs plated at the intervals noted in the table.

Study of table 2 shows that the distribution of the injected typhoid bacilli was, with 2 exceptions, the same for the 4 rabbits receiving the infective dose by way of the blood stream. Comparatively few bacteria appeared in the bile 10 minutes after the injection; the rabbit injected in the ear vein showed the largest number. The liver of this animal removed from the blood stream 9% of the inoculated organism, while

¹⁰ Mitt. a. d. Mediz. Gesellsch. z. Osaka, 1910, 9, refer.; Centralbl. f. Bakteriol. I. Ref., 1911, 50, p. 294.

TABLE 2

FATE OF *B. TYPHOSUS* ON INTRAPORTAL AND INTRAVENOUS INOCULATION COMPARED BY THE SUBCUTANEOUS METHOD OF INJECTION
 Litter Experiment: Intravenous injection of 5,000,000,000 organisms.

Tissue	10 ⁹ Intraportal Injection: 1344	10 ⁹ Intravenous Injection: 1343	24 Hours Intraportal Injection: 1345	24 Hours Intravenous Injection: 1342	24 Hours Subcutaneous Injection: 1349
Left and central liver lobe.....	650,000 (800,000)	810,000 (400,000)	2,400 (3,000)	6,000 (7,400)	0
Right and central liver lobe.....	550,000 (800,000)	600,000 (400,000)	2,000	6,720 (11,000)	0
Bile.....	5 (0.4 c c)	60 (0.2 c c)	0 (1 c c)	0 (0.8 c c)	No gallbladder
Gallbladder wall.....	9,400	1,600	2	128	0
Spleen.....	750,000	2,900,000 (2,310,000)	4,410	1,470	0
Bonemarrow.....	30,000	20,000	23,000,000	410 (380)	0
Mesenteric lymph nodes.....	380	120 (72)	8	20	0
Lungs.....	2,500	6,600	10	136	0
Kidneys.....	450+	590 (420)	0	7	0
Urine.....	0 (1 c c enriched 0)	0 (coeci)	0 (coeci)	0
Testes.....	45
Carotis blood.....	46,000 per c c	125,000 per c c	2 per c c	3 per c c	0
Heart blood.....	8,800 per c c (22,300; 840; 5,000)	18,500 per c c (37,000; 2,900;)	1 per c c	0	0
Brilliant Green Eosin Plates:					
Duodenum contents.....	Sterile	1 colony <i>B. typhosus</i>	7 colonies <i>B. typhosus</i>	Sterile	Sterile green plates
Jejunum wall.....	4 colonies pure	150 <i>B. typhosus</i> pure	Sterile	Negative for <i>B. typhosus</i>	Sterile green plates
Jejunum contents.....	Sterile	2 <i>B. typhosus</i> pure	Sterile	Negative for <i>B. typhosus</i>	Negative for <i>B. typhosus</i>
Jejunum wall.....	4 colonies pure	300 <i>B. typhosus</i> + coli	1 <i>B. typhosus</i> +++	Negative for <i>B. typhosus</i>	Negative for <i>B. typhosus</i>
Ileum contents.....	<i>B. coli</i>	<i>B. aerogenes</i>	800 <i>B. typhosus</i> +++	Negative for <i>B. typhosus</i>	Negative for <i>B. typhosus</i>
Ileum wall.....	3 colonies <i>B. typhosus</i>	3 colonies <i>B. typhosus</i>	1 <i>B. typhosus</i>	Negative for <i>B. typhosus</i>	Negative for <i>B. typhosus</i>
(sacculus rotundatus)	∞ <i>B. aerogenes</i>	+ <i>B. aerogenes</i>		<i>B. coli</i>	
Appendix contents.....	<i>B. coli aerogenes</i>	1 colony <i>B. typhosus</i>	1 <i>B. typhosus</i>	Negative for <i>B. typhosus</i>	
Appendix wall.....	3 colonies <i>B. typhosus</i>	<i>B. coli aerogenes</i>	55 <i>B. typhosus</i> +++	Negative for <i>B. typhosus</i>	
				<i>B. aerogenes</i>	

the intraportal injected animal accumulated only 6% of the total dose. At the end of 24 hours the gallbladder bile was sterile in the remaining 3 animals. More bacilli accumulated in the lung and spleen of the animal injected by way of an ear vein than in the one infected through a portal vein. It is generally known that after an intravenous injection bacteria accumulate in the lung capillaries, and that intraportal injections lead to a saturation of the vessels of the liver. Rabbit 1345 was slightly toxic, which explains the high count in the bonemarrow, as has already been pointed out by Parker and Franke.¹ Microscopically, this tissue showed necroses, innumerable nests of free and clumped typhoid bacilli, and a marked absence of regeneration of polymorphonuclear leukocytes. The saline extract of the liver of this animal was atoxic. Of some interest were the bacterial counts obtained from the intestinal wall of rabbit 1345, in comparison with rabbit 1342. Numerous hemorrhages in the sacculus rotundatus and the appendix wall indicated vascular lesions in the intestinal mucosa and submucosa. The cultures proved the cause of these hemorrhages to be nests of typhoid bacilli, and supported the observations of Ribadeau-Dumas and Harvier,¹¹ and of Chirolanza,¹² that the intestinal content of rabbits can become infected from bacterial emboli and hemorrhages in the intestinal wall. It is obvious that the mesenteric vein inoculation was particularly conducive to the localization of clumped bacilli in the capillaries of the intestines. Under particularly favorable conditions (large infective dose) it is readily possible that the gallbladder wall may become similarly infected. Our particular experiment fails, however, to confirm this conclusion. It is not unlikely that postoperative changes (slight peritonitis, biliary stasis, etc.), which develop as a result of the technic employed to introduce typhoid bacilli by way of the portal system, favor the localization of bacteria in the gallbladder, and they may in part explain the results obtained by Nichols.⁸ The intoxication of rabbit 1345, which was the largest animal of the series, points toward the explanation offered. Moreover, in our experience postoperative starvation is conducive to intoxication, and the formation of foci in the bonemarrow and biliary system. On the other hand, our results differ in no respect from those reported by Carmichael¹³ and by Else.¹⁴ Both experimenters show that organisms injected into the portal vein do not readily reach the gall-

¹¹ *Compt. rend. Soc. de biol.*, 1910, 69, p. 181.

¹² *Ztschr. f. Hyg. u. Infektionskrankh.*, 1909, 62, p. 11.

¹³ *Jour. Path. & Bacteriol.*, 1903, 8, p. 276.

¹⁴ *Surg., Gynec. & Obst.*, 1910, 11, p. 470.

TABLE 3

FATE OF B. TYPHOSUS IN NORMAL RABBITS

Rabbits of the same litter, 5 months old; exsanguinated following injection. Injected intravenously: 4,300,000,000 B. typhosus "Kearney."

Tissue	10*	60†	1 Day	2 Days	3 Days	4 Days	5 Days
	Rabbit 1225	Rabbit 1222	Rabbit 1219	Rabbit 1220 C.	Rabbit 1221	Rabbit 1223	Rabbit 1224
Right liver lobe.....	1,610,000	420,000	700,000	2,320,000‡	180 (139)*	370#
Left liver lobe.....	1,130,000	550,000	10,000?	5,640,000	700	112 (106, 200)	2,133
Center liver lobe.....	1,620,000	380,000	50,400	36,000§	130 (180)	111 (105, 200)	56,000
Gallbladder wall.....	300	310	225,000	64,000	792,000	508,000	112,800
Bile.....	4	1?	D† = sterile; enriched: 0	19,000,000	10,550,000	8,000,000	2,000,000
Spleen.....	per e e			per e e (0.4 e e)	per e e	per e e	per e e
Left bonemarrow femur..	1,280,000	370,000	6,700	1,200	118	245	387
Right bonemarrow femur..	221,000	125,000	1,400	150	13	4 (10)	7
Mesenteric lymph nodes..	190,000	170,000	1,200	330	37	12	3
Lungs.....	250	24	270	67	175?	60	10-21
	13,000 right		{300 right lung } {200 left lung }	70	6	0	0
Left kidney.....	5,700	52	11	0	0	1	0
Right kidney.....	10	24	4	0	2	0
Femoral muscle.....	250	900?	0	0	0	0
Thymus.....	124	70	4
Carotis blood.....	1,000,000 per e e	<100*	1,300 per e e	100 per e e	7 per e e	1 per e e
Right ventricle.....	380,000	Sterile	Sterile
Duodenum.....	6 colonies per loop	0
Sacculus rotundatus.....	0	0
Stool in rectum.....	0	0
Urine.....	Sterile	Sterile	Sterile	50 B. typhosus per loop	2 B. typhosus per loopfull

* Dilution too high.

† D = direct quantitative plating.

‡ Kidney lobe: 760,000.

§ Liver around gallbladder: 1,850 + 3,600.

Kidney lobe of liver: 92.

§ Around gallbladder: 630,000.

bladder. *From an experimental standpoint the mesenteric vein injection offers, therefore, no advantages over the ear vein injection.*

A subcutaneous injection of a large dose of typhoid bacilli fails to cause a general infection. At the end of 24 hours a small number of the introduced organisms can be found at the site of inoculation, while the viscera by ordinary plating procedures are found to be sterile. This experiment confirms similar ones reported by Doerr.¹⁵

The next important step in the analysis of the fate of typhoid bacilli inoculated into rabbits consisted of culturing the tissues of nonimmunized animals over a period of at least five days. Such experiments demonstrated the ability of certain organ tissues to dispose of the accumulated bacteria and supplied data which indicated that a prolonged infection of the bile was associated with an involvement of the liver and the gallbladder wall. One of these instructive experiments is reported herewith.

Expt. 3. The Fate of Typhoid Bacilli in Nonimmunized Rabbits.—Seven rabbits of one litter, with an average weight of 1630 gm., 129 days old, were inoculated into an ear vein with 4,300 million typhoid bacilli strain K., grown on peptic-digest agar. The animals were exsanguinated after 10 and 60 minutes, and after 1, 2, 3, 4 and 5 days, and the excised tissues plated. The results are shown in a concrete form in table 3.

It will be noted from table 3 that within 10 and 60 minutes, even 24 hours, the cystic bile contained very few typhoid bacilli, while the gall-bladder wall recorded for rabbit 1219 a very high count. This observation, together with other data to be reported elsewhere, is in our opinion conclusive evidence that a wall infection frequently precedes the invasion of the bile. Erosion of the capillaries of the gallbladder wall occurred in rabbit 1221. This animal showed a blood-tinged cystic bile with leukocytes and fibrin flakes; the wall itself exhibited patches of necroses and mucosal and subserous hemorrhages. In the other rabbits, aside from a high bacterial count, pronounced vascular changes were not evident, but the liver tissue adjacent to the gallbladder registered a large number of viable bacteria. In the experiment under consideration, at least 48 hours elapsed before the cystic bile was heavily infected, although the hepatic duct bile probably contained typhoid bacilli much earlier as innumerable colonies were present on the plates prepared from the duodenum of rabbit 1219. It may be mere coincidence, but the gradual diminution in the number of bacilli per c c of

¹⁵ Centralbl. f. Bakteriöl., I O., 1905, 39, p. 624.

bile in the rabbits sacrificed on the 4th and 5th day is noteworthy, and may be explained by the more active evacuation of the stagnant biliary secretion, stimulated by the digestive activities of the intestinal tract, which was seriously impaired during the intoxication of the animals in the first 3 days following the injection.

Aside from the points just mentioned, the results recorded for this and similarly conducted experiments did not as a whole differ from those reported by various writers repeatedly mentioned. The liver retained, 10 minutes after the injection, about 20% of the total number of bacteria introduced into the blood stream, while all the other organs, particularly the kidneys, lymph nodes, muscle, etc., retained very few. The bactericidal action of the serums of rabbits 1225 and 1222 was comparatively low; 1 c.c. of serum destroyed in 5 hours only 10,000 typhoid bacilli stain K. This may in part explain the high bacterial count of the blood, and the fact that clinically the animals of this series were in comparison with others only slightly intoxicated. This observation is also supported by the low bonemarrow counts. The speed of destruction is influenced by the resistance of the rabbit, and the number of bacteria inoculated, as is shown by the fact that the liver in rabbit 1222, at the end of one hour following the inoculation, still retained 5% of the total number of bacteria, or about one-fourth of the number of typhoid bacilli which originally lodged in this organ.

Aside from the liver, the gallbladder and its contents, the spleen and bonemarrow may retain viable bacteria at a period when the blood is already sterile, and may remain the site of vegetating typhoid bacilli for from 20-30 days. As a rule, the spleen destroys typhoid bacilli more slowly than the liver, a fact already noticed by Werigo.¹⁶ Simultaneously with the disappearance of the germs from the blood, which depends on the bactericidal substances evolved by the tissues and the number of bacilli injected, the kidneys and lungs are found free, while the mesenteric lymph nodes, probably one account of secondary invasion through lacteals of the intestines, remain more or less infected.

These experiments conclusively demonstrate that the typhoid cholecystitis in normal, healthy rabbits is probably not infrequently the result of capillary bacterial emboli in the gallbladder wall, and that a pericholangitis, or a hepatitis, as has been stated by E. A. Graham¹⁷ is a rather constant accompaniment of this process. This phase of our problem will, however, be considered in the next paper of this series.

¹⁶ Ann. de l'Inst. Pasteur, 1892, 6, p. 478.

¹⁷ Surg., Gynec. & Obst., 1918, 27, p. 521.

A COMPARISON OF THE FATE OF TYPHOID BACILLI IN NORMAL AND IMMUNIZED¹⁸ RABBITS

It has already been stated that Bail, Parker and Franke, and Stone studied the bactericidal powers of normal and immunized rabbits infected by intravenous injections. It has also been pointed out that the results of these workers were obtained by various procedures and that they are not strictly comparable. Bail, for example, worked with rabbits, which were passively immunized by the injection of immune serums. He stated that he encountered considerable difficulty in obtaining uniform results. Parker and Franke prepared their immunized animals with killed and living typhoid bacilli, while Stone inoculated her rabbits with dead bacilli only. Parker and Franke tested their animals with a comparatively small number of typhoid bacilli, while Stone used the profuse growth of blood-agar slants. It naturally suggested itself to investigate whether the various results recorded by these workers were attributable to these differences in technic or to other unknown factors. Our experiments were conducted with these problems in mind, and covered, therefore, three phases: 1. What is the fate of typhoid bacilli intravenously injected into rabbits immunized by dead or living vaccines? 2. Are typhoid bacilli grown on peptic digest agar less readily destroyed by the bactericidal powers of the rabbit's blood and tissues than those grown on rabbit blood agar?² 3. Does the dosage of bacteria injected materially influence the end result, and does the immunized animal really destroy typhoid bacilli better and in a shorter time interval than the normal rabbit? The respective experiments to answer these questions were extensive, and our conclusions were based on several hundred animals. For the sake of brevity an example of each type of test will be selected for consideration and discussion.

Expt. 4. The Fate of Typhoid Bacilli in Normal Rabbits and in Those Immunized by Heat-Killed Tricresolized Vaccines Tested by a Moderately Large Number of Bacteria.—Five rabbits of two litters of 11 animals, with an average weight of 2,090 gm., and about 145 days old, were immunized by six intravenous injections of polyhomogenous heat-killed, tricresolized vaccines (13 and 14). Forty days after the last injection these rabbits, together with 6 control animals, of an average weight of 2297 gm., were infected by an intravenous injection of 3,300 million *B. typhosus* strain K. The rabbits were exsanguinated in pairs after 10 minutes, 5 hours, 1, 2, 4 and 6 days, and the excised organs plated. The counts of viable bacteria are shown in table 4.

¹⁸ Rabbits are immune against the disease typhoid fever, but they can be immunized against the *B. typhosus*. In order to emphasize this difference the term immunized instead of immune is chosen.

TABLE 4

THE FATE OF TYPHOID BACILLI IN NORMAL RABBITS AND IN THOSE IMMUNIZED BY HEAT-KILLED TRICRESOLIZED VACCINES TESTED BY A MODERATELY LARGE DOSE OF BACTERIA

Tissue	10'	10'	5 Hours	24 Hours	24 Hours	48 Hours	48 Hours	96 Hours	96 Hours	144 Hours	144 Hours
	Immune Rabbit 1283	Normal Rabbit 1284	Normal Rabbit 1285	Immune Rabbit 1274	Normal Rabbit 1278	Immune Rabbit 1275	Normal Rabbit 1279	Immune Rabbit 1276	Normal Rabbit 1280	Immune Rabbit 1277	Normal Rabbit 1281
Agglutination.....	1:2000	<1:10	<1:10	1:10,000	<1:10	1:20,000 ++++	<1:10	1:10,000- >1:20,000	<1:10- 1:8,000 ++++	1:8,000- >1:20,000	<1:10 1:4,000
Liver, left and central....	910,000	940,000	4,000-8,000	280	450	210	(8-90)	332	5,800	10	0
Liver, central and right...	1,080,000	830,000	4,500	230	380	270	80	200-260	6,400	8-20	2
Gallbladder wall.....	4,100	730	9	0	2	0	0	0	82,000	0	0
Bile.....	11	0	169	0	0	0	0	0	103,500,000	0	0
	(0.6 c c)	(also en- riched)	(1.6 c c)	(1.8 c c)	(0.5 c c)	(0.6 c c)	(0.9 c c)	(1 c c)	p. bile	(1.3 c c)	
Spleen.....	360,000	230,000	37,000	>3,500	200	201-500-350	104	1,120-1,000	3 (en- riched)	138-240	0
Bone marrow pooled.....	>130,000 +	3,100-5,100	>50,000	1,100	50-80	70	6-8	54	1	4	0
Mesenteric lymph nodes...	280	60	90	2	52	82	6	105-160	0	1	0
Lungs.....	12,000	650	200-1,000	70-100	20	0	0	130-200	0	1	0
Kidneys.....	540	55	17	1	0	0	0	0	910	2,700	0
Urine.....	0	0	200(210 310)	0	0	0	++	33	0
Heart blood.....	0?	100	0	0	0	0	(in 1 c c)	0
Carotis blood.....	750	100	400	0	?	1 in 4 plates	R. & L. V. 1	1 in 3 plates	1 in 3 plates	2 in 3 plates	0
Whole blood clotted.....	230-40-20-72 (5 hours)	10-20-0 (5 hours)	40-20-0 (5 hours)	0	6						
96 Hours											
Normal Rabbit 1280			Immune Rabbit 1277			Immune Rabbit 1277			Normal Rabbit 1281		
Duodenum.....	∞ B. typhosus	Duodenum.....	Duodenum.....	Negative for B. typhosus			Duodenum.....			Negative	
Jejunum.....	∞ B. typhosus	Jejunum.....	Jejunum.....	Negative			Jejunum.....			Negative	
Colon.....	∞ B. typhosus	Colon.....	Colon.....	Negative			Ileum.....			Negative	
Sacculus rotundatus.....	B. typhosus	Sacculus rotundatus.....	Sacculus rotundatus.....	Negative			Sacculus rotundatus.....			Negative	
Stool from rectum, direct.....	Negative	Stool from rectum, direct.....	Stool from rectum, direct.....	Negative			Cecum.....			Negative	
Bile enriched.....	B. typhosus	Bile enriched.....	Bile enriched.....								
Biliary passages.....	4,040,000	Biliary passages.....	Biliary passages.....								
Liver around gallbladder.....	21,200	Liver around gallbladder.....	Liver around gallbladder.....								

Table 4 shows that the ratio of distribution of the typhoid bacilli in the organs of immunized rabbits is practically the same as in the normal animal. In this particular experiment the spleen, bonemarrow and lung of the immunized rabbit sacrificed 10 minutes after the injection, retain more bacteria than the normal. In other experiments this difference is constant only for the lung: This organ may in animals with an acquired immunity seize from 3 to 200 times as many bacteria as that of the normal rabbit. Rather constant is the observation that the splenic tissues of the vaccinated animals retain the typhoid bacilli injected for a longer period, and in somewhat greater number than the normal. These are the only striking differences between the normal and immunized rabbit which have occurred regularly in similarly conducted experiments. The blood serums of the normal rabbits of the two litters exhibited exceedingly high bactericidal properties (see figures on whole blood clotted of rabbit 1284 and rabbit 1285), which in part explains the disappearance of the injected bacteria from the blood stream, irrespective of their low agglutinin content. It is noteworthy that on the sixth day the tissues of normal rabbit 1281 were, with exception of the liver, found sterile, while the immunized animal (rabbit 1277) of the same litter and period of infection, showed typhoid bacilli in most of the organs and even in the blood. The latter finding is probably connected with the persistence of viable organisms in the spleen.

Localization in the gallbladder took place in only one animal (1280). The inflammatory reaction of the gallbladder wall was severe. Indeed, fibrin deposits connected the serosa of the viscus with the stomach wall. An edematous infiltration extended along the cystic duct to the common and to the hepatic biliary passages. Flocculent, slimy débris blocked the common duct and in part explained the high bacterial count of the bile removed from the gallbladder. Judging from these and similar findings, one is again inclined to attribute the cholecystitis to a primary infection of the wall with subsequent invasion of the bile. Attention is also called to the presence of typhoid bacilli in the urine of 3 animals (rabbits 1274, 1280, and 1277). In 2 rabbits this persistence of organisms is associated with a localization of bacteria in the kidneys. Similar observations have indicated that the presence of typhoid bacilli in the urine is frequently characteristic for animals of the same litter, and we therefore suspect an hereditary susceptibility of these organs. More-

over, the typhoid bacilli can multiply in the urines of some rabbits, while in others the findings of these germs is always closely connected with foci in the kidney parenchyma.

The method of immunization, whether accomplished with living or dead organisms, has no influence on the destruction of typhoid bacilli in the blood and tissues of the protected rabbits, as is illustrated by experiment 5, which is again chosen from a number of similar tests.

Exper. 5. The Fate of Typhoid Bacilli in Rabbits Immunized With Living Vaccines and Tested by the Injection of a Moderately Large Dose of Bacteria.—Three members of a litter of 7 rabbits, with an average weight of 1,700 gm., and 132 days old, were immunized according to the method of Bull¹⁹ with a mixture of 4 strains (Bl., B., K. and I.) of living typhoid bacilli. Twenty-eight days after the last injection these rabbits, together with the 4 controls of the same litter, were injected intravenously with 4.100 million of a *B. typhosus* mixture (strain Bl., B., K., and I.). The distribution and localization of the injected bacteria were studied in one normal rabbit. The remaining 6 animals were sacrificed in pairs after 8, 24, and 72 hours, and the organs plated. The observations are summarized in table 5.

A study of table 5 shows that the serums of the nonimmunized members of this litter destroyed typhoid bacilli rather slowly. The immunization rendered the blood nonbacteriolytic in the test tube, and yet in 2 rabbits killed on the 24th and 72nd hour after the injection, the blood stream harbored fewer organisms than in the normal animals (3 and 1 to 44 and 75). On the other hand, the splenic pulp of the immunized rabbit retained from 2 to 20 times as many bacilli as the normal. The lung tissue of the immunized animal retained on the 8th hour 3 times as many bacteria as its liver, and 60 times as many as the lung of the normal control rabbit. Gallbladder infections resulted in only one normal animal. The number of organisms determined in 1.6 c c of bile was 490; the gallbladder wall registered 31 colonies per 100 mg. of tissue. This particular animal showed numerous infarcts in the liver, and in comparison with other rabbits it was obvious that the presence of a small number of typhoid bacilli was probably the result of biliary elimination from these liver foci. Both the normal and immunized rabbits sacrificed on the 8th hour had bacilli in the bile (10 and 36 per 1 c c). This and similar observations indicate that the mere presence of typhoid bacilli in the gallbladder bile does not lead to a prolonged infection of this viscus.

¹⁹ Jour. Exper. Med., 1916, 23, p. 419.

TABLE 5

THE FATE OF TYPHOID BACILLI IN RABBITS IMMUNIZED WITH LIVING VACCINES AND TESTED BY THE INJECTION OF A MODERATELY LARGE DOSE OF BACTERIA

Tissue	10 ⁷ Normal Rabbit 1327 B	8 Hours Immune Rabbit 1327	8 Hours Normal Rabbit 1329	24 Hours Immune Rabbit 1328	24 Hours Normal Rabbit 1332	72 Hours Immune Rabbit 1330	72 Hours Normal Rabbit 1331
Agglutination.....	<1:10	1:10,000	1:10	1:8,000	1:10	1:8,000-10,000	1:60-100
Left and central liver....	780,000	42,000	60,000	1,300	7,000	62	780 / In-
Right and central liver....	750,000	36,000	120,000	1,400	8,100	46	1,200 (fretts
Gallbladder wall.....	5,500	180	220	0	10	0	31
Bile.....	36 per c c	10 per c c	0	(cocci)	0	(cocci)	490 per c c
Spleen.....	(1.0 e c)	(1.1 e c)	(1.2 e c)	0	(1.2 e c)	0	(1.6 e c)
Bonemarrow.....	1,610,000	20,000	1,100	4,700	4,100	6,100	2,500
Mesenteric lymph nodes...	364,000	37,000	40,000	2,500	8,000	20	190
Lungs.....	1,700	152	80	152	95	160	300
	10,000	107,000	1,680	750	300	2	1
Kidneys.....	13,200	2,800-3,200	170	19	10	(B. bronchi-	(B. bronchi-
Urine.....	0 (3)	0	0 (3)	0 (3)	0 (1)	<10	septicus)
Thymus.....	1,050	2
Carotis blood	I 2,325,000 per c c	I 265 per c c	I 105 per c c	I 11 per c c	I 44 per c c	0, 0, 1 per c c	I 74 per c c
	II 1,620,000 per c c	II 225 per c c	II 85 per c c	II 3 per c c	II 39 per c c	0 per c c	II 56 per c c
Heart blood.....	Negative for	Negative for	Negative for	Negative for	Negative for	Negative for
Duodenum.....	1 colony of	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus
	B. typhosus	Negative for	Negative for	Negative for	Negative for	Negative for	Negative for
Jejunum.....	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus
	Negative for	Negative for	Negative for	Negative for	Negative for	Negative for
Ileum.....	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus
	Negative for	Negative for	Negative for	Negative for	Negative for	Negative for
Cecum.....	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus
	Negative for	Negative for	Negative for	Negative for	Negative for	Negative for
Appendix wall.....	2 colonies	61 B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus	B. typhosus
	colonies	Negative for	Negative for	Negative for	Negative for	Negative for
Appendix contents.....	Negative for	Negative for	Negative for	Negative for	2 B. typhosus	Negative for	Negative for
	B. typhosus	B. typhosus	B. typhosus	B. typhosus	ulcer in colon	Negative for	Negative for
					∞ B. typhosus	B. typhosus	B. typhosus

A bacilluria of 3,400,000 typhoid organisms per c.c. with a few bacteria in the kidneys (2 per 100 mg.) was noted in a normal rabbit killed on the third day. The liver of the normal rabbit killed on the 8th hour contained foxins demonstrable by the method of Parker, while the saline extract of the same organ of the immunized animal was innocuous. On several occasions we tested the fate and destruction of typhoid bacilli in rabbits which were gallbladder-typhoid carriers. The removal of the organisms from the blood by the tissues, and their bactericidal action proceeded in these animals in an identical manner, as observed in control rabbits studied at the same time under identical conditions.

The experiments of Parker and Franke were conducted on rabbits immunized with living typhoid bacilli, while Stone tested animals vaccinated with heat-killed preparations. The only additional differences in the experiments of these workers was the use by Stone of typhoid bacilli grown on rabbit-blood agar slants. Typhoid bacilli acquired on this medium, particularly when transplanted at regular intervals of from 24-48 hours, certain properties which apparently are conducive of gallbladder infections. Physical, as well as biologic factors, which we have not been able to study with the desired thoroughness, are probably responsible for these properties. The growth energy of *B. typhosus* is enhanced on blood mediums and with it also the pathogenic powers, particularly the parasitic function. This applies in a lesser degree to the toxicogenic properties. In many respects this behavior of typhoid bacilli frequently transferred on blood mediums is analogous to the changes in virulence observed by Wadsworth and Kirkbride²⁰ for the pneumococcus. From several experimental series in each of which 2 normal rabbits were infected with typhoid bacilli grown on rabbit-blood agar (35 generations) and 2 others with the same number of bacteria reared on veal-infusion agar (same number of generations), it must be concluded that no difference in the distribution and localization or destruction of the germs is demonstrable. Toxic symptoms and a high bonemarrow count at the end of the 24th hour were, however, noted in several animals injected with the veal-agar growth. These observations suggest a number of tests on immunized rabbits and one experiment of a series of 3 is chosen for discussion.

²⁰ *Ibid.*, 1918, 28, p. 791.

TABLE 6
THE FATE OF B. TYPHOSUS GROWN ON RABBIT-BLOOD AGAR IN THE IMMUNIZED AND NORMAL RABBIT

Tissues	10 ⁷ Normal Rabbit 1595 o +	24 Hours Immune Rabbit 1646 o	24 Hours Normal Rabbit 1522 o +	96 Hours Immune Rabbit 1591	96 Hours Normal Rabbit 1686*	120 Hours Immune Rabbit 1589	120 Hours Normal Rabbit 1593
Agglutination titer.....	1:10+++	1:40,000	1:10	1:60,000++++	1:40++++	1:40,000++++	1:800++++
Left and central liver....	1,200,000	840	300	30,000	800,000	60,000	1,600
Right and central liver....	2,000,000	760	1,210	1,100	840,000	50,000	630
Bile.....	1,334 (0.5 e c)	0 (enriched +) 0.05 e c	0 (1.2 e c)	1,420,000 (0.9 e c)	940,000,000 per 0.8 e c	58,000,000 per 0.7 e c	50,000 per 0.8 e c
Gallbladder.....	7,200	10	0 difco streptococci	19,000	5,500,000	230,000	44,000
Spleen.....	900,000	1,500	400	24	17,000	1,600	410
Bonemarrow.....	80,000	5,500	5,000	36	6,000,000	24	58
Mesenteric lymph nodes...	15,000	17	18	22	3	21
Lungs.....	22,000	7,000	3,000	250	1,800	150	7
Kidneys.....	62,000	9	3	180	267,000	0 (enriched + +)	12,000
Urine.....	3, 2 per e c	0, 0	169 per e c	0; 0	10	0	66,000 per e c
Carotis blood.....	4,800,000; 6,000,000	35; 20; 14; 20; 23; 14	76; 65; 140; 55; 90	0; 0; 0	0; 0; 0; 0; 0	0; 0; 1; 0; 1; 0
Heart blood, left vent.	4,900,000	12; 10	66; 40	0; 0; 0	8 partially coagulated blood	0; 0; 0	3; 0; 0
right vent.	3,000,000	20; 14	40; 130; 90	0; 0; 0	0; 0; 0	0; 0; 1
Duodenum.....	6 colonies pure	Negative for B. typhosus	Negative for B. typhosus	∞ B. typhosus colonies	∞ B. typhosus colonies	5 B. typhosus colonies	∞ B. typhosus
Sacculus rotundatus.....	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	1 B. typhosus colony	∞ B. typhosus colonies	8 B. typhosus colonies	Negative for B. typhosus

* Died shortly before cultures were made.

Expcr. 6. The Fate of B. Typhosus Grown on Rabbit-Blood Agar in the Immunized and Normal Rabbit.—Three rabbits of a litter of 7 animals, with an average weight of 2,500 gm., and about 170 days old, were immunized with a mixture of 7 strains of heat-killed and living typhoid bacilli in a similar manner as that described in exper. 5. Fifteen days after the last injection these animals, in addition to the remaining 4 members of the litter, were infected by an intravenous injection of 4,300 million of a polyhomogenous saline mixture of 7 typhoid strains grown on 10% rabbit-blood agar. The cultural findings are shown in a concrete form in table 6.

Attention is called to the fact that the immunized and normal rabbits exhibited pronounced localization of the typhoid bacillus in the gall-bladder and bile 96 and 120 hours after the introduction of the infective dose, while the animals killed on the 24th hour gave sterile cultures from this viscus. These observations indicate that blood-agar cultures are suited for the production of biliary infections, but they also confirm the conclusion reached from exper. 3, that the bile or the gallbladder does not necessarily become infected in the first 24 hours. It may also be assumed that elimination of typhoid bacilli by the hepatic duct bile in the first 10 minutes does not necessarily lead to a prolonged infection of this viscus. The evidence in this and similar experiments is decidedly in favor of the conception that, in the rabbit intravenously inoculated, not infrequently an infection of the gallbladder wall, or extensive liver lesions, initiate the invasion of the bile. It should, however, be emphasized that in this respect no difference exists between the immunized and normal rabbits. Anatomically, hemorrhages and even slight erosions of the gallbladder mucosa are distinctly visible; every rabbit shows, also, extensive liver necroses. In rabbit 1589 an obstruction of the common duct produced a uniform dilatation of the bile ducts which explained the high bacterial count of the liver tissue, while in rabbit 1593 the washed gallbladder wall registered practically the same number of typhoid bacilli as 0.8 c c of bile.

The bacterial count of the bonemarrow of rabbit 1686 indicates the existence of a focus, which led, directly or indirectly, to the intoxication and to the death of the animal. The high bacterial counts of the tissues must be partially attributed to a multiplication of the typhoid bacilli in the blood stream during a rather prolonged agonal stage of the disease, and partially to the fact that toward the termination of the infection the organs, like the liver, the spleen and the lungs, primarily engaged in the destruction of the bacteria, are overwhelmed with organisms brought to them from the bonemarrow foci. It is known from the statements already made that 3-4 days usually elapse before the blood of the normal,

and frequently also the immunized, rabbits is found sterile. In our experience with a considerably larger number of animals than reported by Stone, we have been unable to confirm her conclusions that the blood of typhoid immunized rabbits sterilizes itself in 18 hours. Heart-blood cultures, of course, should be chosen for this comparison. In rabbits showing extensive liver or splenic foci the carotid blood toward the end of the exsanguination may record a few typhoid bacilli. Furthermore, sterility of the blood is in no way dependent on its content of immune agglutinins or other antibodies. Rabbits immunized with sensitized bacteria, as a rule, lack agglutinins, although they are similarly resistant to an infection as are rabbits protected with living bacteria.

Most of the rabbits in this experiment possessed at necropsy a distinct splenic tumor, and this tissue in the immunized rabbits retained slightly more bacilli per 100 mg. of organ than the normal ones. For reasons already given, the pair of rabbits killed on the 96th hour cannot be included in these deductions.

Localization in the kidneys, early elimination, and even persistence of the typhoid bacilli in the urine, were characteristic for this litter. The latter condition might be due in part to a change in the reaction and composition of the urine. Complete disappearance of the urinary carbonates and a distinct reduction in the alkali blood reserve occurred in the rabbits with urinary infections.

Briefly summarized, it can be stated that the intravenous injection of normal and immunized rabbits with typhoid bacilli grown on rabbit-blood agar failed to demonstrate differences in the destruction and the distribution of the bacteria. It was, however, noted that localization in the gallbladder and bile 96-120 hours after the injection occurred more readily with moderately heavy suspensions prepared from rabbit-blood agar slants than with those cultivated on peptic-digest agar.

In accordance with the plan of our investigation it was necessary to verify the interpretations of Stone: Can an immunized rabbit dispose more rapidly of a small number of typhoid bacilli than a nonimmunized one; and are the results published by Parker and Franke really explained by the fact that these workers employed as an infective dose less than 1 billion bacteria? In three series of experiments these questions were investigated. The number of organisms injected varied from 215-562 million bacteria. Litters of different ages were chosen, and it was conclusively demonstrated that no differences existed in the normal or immunized rabbits, either in the destruction or in the localization of

such numbers of typhoid bacilli. Two hundred and fifteen million were disposed of in a shorter time interval than 562 million, but the immunized animal differed in this respect in no way from the normal. Moreover, the variable resistance of different litters, sometimes influenced by *B. bronchisepticus* infections, was more readily demonstrable following the injection of small doses of typhoid bacilli, but in no case were these variations greater in immunized than in normal animals. The same deduction applies to the apparent differences depending on the toxicity or virulence of the individual typhoid strains. The correctness of these conclusions is illustrated by the description of one experiment.

Exper. 7. The Fate of a Small Dose of B. Typhosus in Rabbits Immunized With Dead and Living Organisms.—Three rabbits of one litter and 3 of another, all 197 days old, and of an average weight of 1,991 gm., were immunized either by Bull's method with a polyhomogenous mixture of typhoid bacilli, or by 6 injections of a heat-killed, tricresolized vaccine. Seventeen days after the last injection, these rabbits, together with 3 controls (members of the litter immunized with dead bacteria) were injected with 350 million typhoid bacilli (7 strains). The cultivating of the organs was conducted as usual. Table 7 presents the results in concrete form.

The figures presented in table 7 seem to indicate that the number of typhoid bacilli inoculated has no appreciable influence on the mechanism responsible for their progressive destruction in the rabbit body. It may be mere coincidence, but in this and similar experiments, it was noted that frequently the normal rabbit sterilized itself in a shorter time interval than the immunized animal. The bacterial counts of the spleen and bonemarrow should be chosen for such comparison. In the experiments under consideration, Rabbit 1644, possessing a gallbladder infection probably contracted during the immunization with living organisms, disposed rather slowly of the inoculated bacteria. Furthermore, experiments suggest that the blood stream will remain free from viable typhoid bacilli when the spleen has completely disposed of the accumulated microbes. Infarction of the spleen with marked enlargement and a poor exsanguination are some of the factors responsible for the high bacterial count in rabbit 1588.

Certain observations pertaining to the localization of the inoculated bacilli deserve some brief consideration. The liver retains in the first 10 minutes after inoculation from 10 to 20% of the injected organisms. The figures dealing with the lung tissues are also of interest. The lungs of rabbit 1647, immunized with living bacteria, removed from the blood stream about 100 times as many bacilli as the normal, and 10

TABLE 7
THE FATE OF A SMALL DOSE OF *B. TYPHOSUS* IN RABBITS IMMUNIZED WITH DEAD AND LIVING ORGANISMS

[illegible]

times as many as rabbit 1580, which was only treated with heat-killed vaccines. As a rule, this behavior of the lung tissue is constant in healthy, immunized rabbits, but may be seriously disturbed by a latent *B. bronchisepticus* infection, so frequently encountered in animals subjected to bacterial vaccines or their split products. Extensive immunization, however, is conducive to a deposition of bacteria in the pulmonary capillaries. Just why they are removed by the lung of the immunized or sensitized animal we have not as yet determined conclusively, but the recent observations of Coca²¹ on sensitized rabbits suggest that an occlusion of the pulmonary vessels is perhaps the most important factor. Most of the investigators dealing with the distribution of bacteria in the normal lung of animals following an intravenous injection conclude that the organisms accumulate in these tissues as a result of mechanical influences, notably the difficulty of dispersing the bacteria in the blood, the small size of the capillaries and the secondary accumulation of leukocytes. According to our observations, neither the dispersion nor the accumulation of leukocytes is notably different in the immunized rabbits, but the lumen of the capillaries is narrowed and assists in the retention of the injected organisms.

In comparing our data with those published by Parker and Franke, we note that as a whole the results of the experiments on immunized and normal rabbits are identical. However, certain figures in their article appear to be incorrect. For example, on page 306 they state that a normal rabbit (1), weighing 1,900 gm., was injected with 39,627,000 typhoid bacilli; the liver retained 15 minutes after the inoculation 270,000 bacteria per 100 mg. of tissue; calculated for the liver weight²² of this animal, this organ alone would have retained 153,000,000 bacteria. A similar discrepancy exists between the inoculum and the liver count for an immune rabbit 2. The inoculation was stated to be 39 million typhoid bacilli, while based on calculations, the liver removed in 10 minutes 287 million. Throughout this publication the percentage of bacteria retained by the liver tissue varies between 30-60%. These figures are, according to the data furnished by Arima²³ and our own, unquestionably too high, and it appears not unlikely that the counts dealing with the test dose are incorrect. The more recent work of Stone is similarly invalidated by incorrect determinations of the inoculated bacteria. According to her estimates, 460,000 million, or 460 billion

²¹ Jour. Immunol., 1919, 4, p. 219.

²² We have estimated the liver weight of 200 normal rabbits as 1/30 of the body weight.

²³ Arch. f. Hyg., 1911, 73, p. 265.

were inoculated, and yet at the end of 5 hours the blood stream of one rabbit contained only 30 bacteria per c c. In view of these discrepancies, it is justifiable to assume that in all probability not more than 4,600 million bacteria were actually inoculated. Moreover, in the light of the data of Bail³ and our own, it appears utterly impossible that the blood and tissues of an immunized rabbit can destroy in 18-24 hours 460 billion typhoid bacilli. The findings of Stone are certainly "paradoxical," to use her own expression, but one is unable to explain the sources of error on account of the sketchy character of the experimental data. It is not unlikely that the method of incomplete tissue cultivation or the preparation of the tissue suspensions is at fault.

The results of our experiments with typhoid bacilli intravenously inoculated into normal and immunized rabbits can be briefly summarized:

1. A small number of bacteria is rapidly removed from the blood stream; about 20 to 30% of the inoculum can be found in the liver, a smaller percentage in the spleen, bonemarrow and lungs, and comparatively small numbers in the lymph nodes, kidneys, muscle, etc. The gallbladder bile contains a small number of bacteria, while the gallbladder wall receives, according to its size, a proportional share of the total number of bacteria deposited in the liver. The intraportal injection has no advantage over the intravenous method.

2. Large numbers of typhoid bacilli are less speedily removed from the blood stream, but the distribution in the organs remains the same in the first 4 to 8 hours. The bacteria taken up by the tissues are rapidly reduced in number, particularly in the liver, spleen and lungs. The bonemarrow is less active and not infrequently the typhoid bacilli multiply in these tissues and form foci, which reinfect the blood stream. The gallbladder can become infected either as a result of an immediate extensive elimination of the introduced bacteria, or the continuous discharge of bacilli from liver foci in the hepatic bile, or in consequence of an embolic infarction of the gallbladder capillaries, causing a diphtheric cholecystitis. In the latter instance the cystic bile may not become invaded with typhoid bacilli for 24-48 hours after the intravenous injection.

3. The distribution and destruction of small or large doses of typhoid bacilli is practically the same in the normal as in the immunized rabbit, whether protected by the injection of dead or living organisms.

The only noteworthy difference between the normal and immunized rabbit is found in the lung and spleen; namely, the lung of the immunized rabbit takes up a somewhat greater number of bacteria than that of the normal; and the spleen of the immune sterilizes itself more slowly than that of the normal.

4. Localization in the gallbladder and bile 96-120 hours after the infection, occurs more readily with moderately heavy suspensions prepared from rabbit-blood agar than with those obtained from peptic-digest agar slants.

5. Rabbits which succumb to the typhoid intoxication regularly harbor enormous numbers of typhoid bacilli in the bonemarrow of the long bones; these foci are probably the seedbeds responsible for the continuous invasion of the blood stream and the subsequent overwhelming infection of the liver and spleen.

THE LOCALIZATION AND SUBSEQUENT DESTRUCTION OF PARATYPHOID A BACILLI IN THE TISSUES OF NORMAL AND IMMUNIZED RABBITS INFECTED BY THE INTRAVENOUS ROUTE

It has been stated in the introduction that the paratyphoid A bacillus behaves like the typhoid bacillus in the tissues of rabbits. This conclusion is based on 3 series of experiments—2 conducted with a large number of bacilli (4,500 million and 3,400 million, respectively); and one with a moderately large inoculum (590 million). One experiment is chosen for discussion.

Exper. 8. Five rabbits of 2 litters, about 210 days old, and an average weight of 2,427 gm., were immunized by Bull's method (dead and living bacteria) with a mixture of 3 paratyphoid A strains, isolated from carriers and obtained through the courtesy of Dr. Charles Krumwiede from the New York City Department of Health. Fifteen days after the last injection these rabbits, with an equal number of controls, members of the same litters, were injected intravenously with 3,400 million of a mixture of the paratyphoid A bacilli used for the immunization. After the lapse of 10 minutes, 24, 48, 96 and 144 hours the rabbits were exsanguinated, their organs excised and cultures made by the usual technic.

Two normal and one immunized rabbits showed extensive biliary infections, with correspondingly high counts in the liver tissue. The counts in the splenic tissue and bonemarrow of the normal, as well as the immunized, rabbits were low and practically identical; for example, the spleen cultures showed on the 48th hour for the normal rabbit 79,

and the immunized, 70 colonies per 100 mg. of tissue; on the 96th hour for the normal, 106, and 200 for the immunized.

The lung tissue of the immunized rabbit retained 200 times the number of bacteria deposited in the same tissue of the normal. The blood stream of the animals was not sterile before the 96th hour; at the end of 24 hours the blood of the normal animal registered fewer bacteria than the immunized rabbit. Five animals, or 50%, exhibited either renal or urinary bladder infections; the urine of one immune rabbit contained 3,300 paratyphoid bacilli per c c.

The results obtained in these experiments differed in no way from those reported for the typhoid bacillus. The well-known native typhoid immunity, which is apparently responsible for these results in rabbits, is likewise potent for the paratyphoid A. bacillus. The conclusions which have been drawn from the experiments with the typhoid bacillus can be applied unreservedly to the studies conducted with the paratyphoid A bacillus.

THE LOCALIZATION AND SUBSEQUENT DESTRUCTION OF PARATYPHOID B
BACILLI (RABBIT AND HUMAN ORIGIN) IN THE TISSUES OF
NORMAL AND IMMUNIZED RABBITS INFECTED
BY THE INTRAVENOUS ROUTE

It is well known that rabbits may be highly susceptible to paratyphoid B bacilli. One of us (K.F.M.²⁴) discussed this subject recently in connection with the description of a spontaneous paratyphoid B epidemic in laboratory rabbits. The isolation of a rabbit-pathogenic organism suggested some experiments along the lines already mentioned for the typhoid and paratyphoid A bacilli. In the course of these tests it was noted that the bactericidal powers of the immunized rabbits excelled those of normal rabbits infected with moderately large doses of virulent animal paratyphoid bacilli. In general the same principle was found to hold true for human paratyphoid B bacilli or *B. schottmülleri*. One experiment is chosen to discuss this important phenomenon.

Exper. 9. Ten rabbits of 2 litters over 1 year old, of an average weight of 3,080 gm., were used. Five animals were immunized by 3 subcutaneous injections of 0.01, 0.05 and 0.1 c c of a 24-hour old living broth culture of *B. aertryckei* (strain 1371).²⁴ Twenty-eight days after the last injection, the serum of these rabbits agglutinated the immunizing strain in dilutions of 1:1000 to 1:10,000, while the nonprotected animals of the same species either failed to clump the

²⁴ Litch and Meyer, Jour. Infect. Dis., 1920, 28, p. 27.

antigen, or did so in dilutions not exceeding 1:20. The entire series of rabbits was infected by an intravenous injection of 700 million bacteria of the identical strain, 1371. Exsanguination, excision of the organs and plating were conducted 10 minutes, 4, 24, and 72 hours after the introduction of the infective dose. The remaining normal animal was moribund on the 120th hour and was killed; while cultures were made from the immunized rabbit on the 144th hour. The cultural findings are presented in concrete form in table 8.

It is clearly demonstrated in table 8 that the immunized rabbits possess the power to destroy paratyphoid B. bacilli more readily than the nonprotected animals of the same species. These differences are clearly evidenced by the clinical symptoms; the immunized rabbits appear lively and lose little weight, while the normal ones, after an incubation time of at least 24 hours, refuse to eat, exhibit general prostration, labored breathing, severe diarrhea, and loss of from 300 to 500 gm. of body weight. In contrast to typhoid animals in which the evidences of intoxication occur early, these symptoms never develop before the 24th hour. Aside from this striking fundamental difference, there are several other features which distinguish a paratyphoid B infection from the typhoid infection in the rabbit, hence a detailed analysis of the data presented in table 8 is justified.

The ratio of distribution of paratyphoid bacilli in the organs is practically the same as that noted for the typhoid bacillus; again the lung tissues of the immunized animals retain slightly more (2-7 times) organisms than the normal. The predominance of bacteria in the normal spleen is rather constant; this organ not only retains a large number of bacteria, but is frequently unable to dispose of the invaders. In case of recovery from the infection, 15 to 25 days elapse until sterility is established. On the other hand, the spleen of the immunized rabbits becomes free from bacteria in from 6-8 days if less than one billion micro-organisms has been inoculated. Occasional infarction will prolong this period for 10 or more days. These observations furthermore suggest that the persistence of a large number of paratyphoid bacilli in the spleen is in all probability responsible for the organisms in the blood stream. Sterility of the blood in rabbit 1415 was accompanied also by sterility of the spleen and bonemarrow, and was not due to the agglutinin content of the serum. In fact, the importance of the spleen was demonstrated even more distinctly in several additional experiments not reported in this paper. Invariably the number of bacteria found in this organ in normal rabbits shortly before death exceeded that recorded for the liver or bonemarrow. For example, in a moribund rabbit, 1477,

TABLE 8
THE LOCALIZATION AND SUBSEQUENT DESTRUCTION OF PARATYPHOID B. RACILLI (RABBIT ORIGIN) IN THE TISSUES OF NORMAL AND IMMUNIZED RABBITS INFECTED BY THE INTRAVENOUS ROUTE

Tissues	Immune Rabbit 1322 Agglutination 1:8,000		Normal Rabbit 1443 Agglutination 1:60		Immune Rabbit 1333 4 Hours in Vivo	Normal Rabbit 1445 4 Hours in Vivo	Immune Rabbit 1416 24 Hours	Normal Rabbit 1447 24 Hours	Immune Rabbit 1444 72 Hours	Normal Rabbit 1444 72 Hours	Immune Rabbit 1415 144 Hours	Normal Rabbit 1446 120 Hours
	10 ⁷	4 Hours' Incubation	10 ⁷	4 Hours' Incubation								
Left and central liver.....	111,000	3,400,000	148,000	14,000,000	1:1,000 3+- 1:4,000 2+- 11,700	118,000	1:8,000 3+-	45,000	1:6,000 3+-	25,500	1:8,000- 1:10,000	27,000
Right and central liver.....	102,000	4,000,000	170,000	11,400	95,000	3,400	3,400	41,000	4,000	28,900	570	134,000
Gall bladder wall.....	136	288	38	20	13	13	80	2,850	10	300,000	18,500,000
Bile.....	0	257	44,200	0	0	0	3,800	1,500	1	270,000,000	18,900,000
Spleen.....	6,700	6,200,000	186,000	2,350	(0.45 c c)	(0.6 c c)	(0.6 c c)	(1.1 c c)	(0.7 c c)	(1.13 c c)	(0.5 c c pus)	165,000
Bone marrow.....	14,100	1,110,000	22,000	8,000	23,000	135	135	219,000	5,400	130,000	1	105,000
Mesenteric lymph nodes.....	42	2,180	38	12	43,000	450	450	52,000,000	550	21,300	0	100,000
Lungs.....	12,500	24,000	5,000	342	30,000	62	62	190	5,000	42,300	36	960
Kidneys.....	230	43,000	1,290	40	312	130	130	16,000	152	21,800	0	25,000
Urine.....	0; 0	0; 0	57	(76)	4	132,000	(sterile)	80,000
Carotis blood.....	per c c	per c c	per c c	11; 15; 8	per c c	3,500; 4,100;	3 per c c	97, 142	per c c	10,000
.....	800; 2,800	11,000;	6; 2; 4	40; 50; 56;	3,400 per c c	per c c	per c c
.....	per c c	9,500 per c c	per c c	63 per c c
Clotted blood.....	220	136	2,300	600,000	13 per c c	4,000 per c c	6, 70	690, 480,	600; 2,100;
Heart blood.....	per c c	1,200 per c c	0 per c c	680 per c c
Contents of:.....	Sterile	Sterile	Neg. for B. para-	Neg. for B. para-	Neg. for B. para-
Duodenum.....	Sterile	1 B. paratyphoid colony	Sterile on green plate	Sterile on green plate	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B	1 colony B. paratyphoid B	Neg. for B. paratyphoid B
Jejunum.....	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B
Ileum.....	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B
Sacculus rotundatus.....	1,000 B. paratyphoid B	10 B. paratyphoid B	Neg. for B. paratyphoid B
Appendix.....	33 B. paratyphoid B	200 B. paratyphoid B	Neg. for B. paratyphoid B

exsanguinated on the 40th hour, the spleen contained 88 million paratyphoid bacilli per 100 mg., the bonemarrow 22 million, the liver 11 million, and the blood 75,000 bacteria. The activities of the spleen are further indicated by the anatomic response of the organ; a spleen tumor, together with an increased weight, is rather constant. The average splenic weight for the immunized animal was 2.61 gm., and for the normal, 2.15 gm., in certain individual cases a weight of 4.25 and 3.36 gm., respectively, was recorded. Compared with the normal weight and estimated per 1,000 gm. of bodyweight, it was calculated that the increase in weight is more marked in the immunized than in normal rabbits, which in turn suggests an increase in cellular elements. It has been shown by F. A. Evans²⁵ that the acute splenic tumor of rabbits infected with paratyphoid-like bacteria (*B. enteritidis*) is characterized by hyperplasia of the phagocytic reticulo-endothelial macrophages, and by a decrease in the number of the other cells of the pulp. The latter condition is probably the result of a toxic inhibition of the leukopoietic function and perhaps a stimulation of the functional reticulo-endothelial cells. The lymphatic nodes also exhibit, in certain rabbits, remarkable affinity for the paratyphoid bacilli, the significance of which fact is not quite clear, but deserves further elucidation. In the first 24 hours the differences between immunized and normal rabbits are fairly uniform; the blood, liver and spleen of the latter contain 10 times as many living organisms as the former. For the lung tissue this contrast is not so clearly demonstrated on account of the initial high count. After 24 hours the actual number of bacteria demonstrable in the organs of the individual rabbits may fluctuate considerably and less uniform counts are obtainable. The focal necroses and embolic processes (gallbladder), which develop quite frequently in immunized animals, and the variations in the individual resistance even in litters, are conducive to these differences. Focal necroses in the bonemarrow of rabbit 1447 and bacterial emboli indicated by hemorrhages in the kidney cortex of the same rabbit, as well as in rabbit 1444 and rabbit 1446, are probably responsible for the high bacterial counts recorded.

Two normal and 2 immunized rabbits developed biliary infections. The finding of paratyphoid bacilli in the bile of 2 animals in the first 24 hours is probably the result of an elimination of these organisms by way of the biliary passages, and not the sequel of a gallbladder wall infection, as noted for rabbit 1415 and rabbit 1446. This phase of the

²⁵ Bull. Johns Hopkins Hosp., 1916, 27, p. 356.

problem has already been discussed by Litch and Meyer in a recent paper on rabbit typhoid (100). These observations likewise confirm the statement that rabbits intravenously inoculated may fail to register bacteria in the bile 24-72 hours after an intravenous injection, and yet may develop a chronic cholecystitis as a result of bacterial embolism of the gallbladder wall. Every nonimmunized rabbit which succumbed to the intravenous infection on the 4th-11th day exhibited a diphtheric, necrotized cholecystitis, although repeated observations in supplementary tests failed to indicate that paratyphoid bacilli are discharged through the bile stream in the first 30 minutes following an intravenous injection of a number of bacteria large enough to insure a general infection and death in from 3 to 5 days. After a lapse of 24 hours bacteria are undoubtedly eliminated by the bile, as is suggested by the positive cultures obtained from the duodenal contents of rabbit 1444, while the gallbladder is sterile, or nearly so, and the wall of this viscus contains a few bacteria corresponding entirely to the degree of the general blood infection. In this connection another difference between the typhoid and paratyphoid infection of the rabbit is noted. The contents of the ileum, sacculus rotundatus and appendix of the typhoid rabbits never demonstrated specific bacteria without positive findings in the duodenum and jejunum, while in several animals (rabbits 1353, 1416 and 1447) of exper. 8, paratyphoid bacilli could be demonstrated in the above mentioned portions of the intestinal tract without receiving their seedings from the upper segments or the bile. It is not unlikely that the constant involvement of the lymphatic structures of the intestines of paratyphoid rabbits leads to a discharge of specific bacteria into the intestinal content, producing the positive cultures recorded. Besredka²⁶ has recently called attention to this localization of Shiga dysentery and paratyphoid bacilli in the large intestines of rabbits and, based on these observations, he has developed an attractive theory on the local immunity of the intestinal mucosa to specific bacterial invasion. In connection with the discussion of our feeding experiments with organisms of the typhoid-paratyphoid group, this subject will be considered.

It has already been stated that the observations recorded in the foregoing were confirmed by several experiments with human paratyphoid strains. As distinct serologic differences have been demonstrated between these strains and true *B. schottmülleri* by numerous independent

²⁶ Ann. de l'Inst. Pasteur, 1919, 33, pp. 301 and 557.

workers, and as it is as yet undecided whether the paratyphoid infection in man is identical with "rabbit typhoid," we repeated our immunity tests with several strains of *B. schottmülleri* isolated from typical human cases and sent to us by Mr. Harry Weiss of the Department of Bacteriology, Columbia University. Using a test inoculum (1-2 billion), which caused death after 6 to 8 days in the nonimmunized rabbits, the results were practically identical with those reported for the animal strains. An ability to destroy paratyphoid B bacilli more quickly is also evidenced in the immunized animals when a small number (300 million) of bacilli is intravenously inoculated; in fact, this phenomenon is particularly striking for the blood. To dispose of a number of organisms which can be destroyed by a normal animal in 5-6 days, an immune rabbit requires less than 24 hours. But in no instance was a cholecystitis produced: the number of bacilli injected was not sufficient even to cause an early discharge in the hepatic duct bile.

In conclusion, it may be stated that the mechanism of destruction of the paratyphoid B bacilli when intravenously inoculated into immunized and normal rabbits differs from that constantly observed for the typhoid bacillus. This applies whether one uses *B. aertryckei* of rabbit origin or *B. schottmülleri*. Paratyphoid B. bacilli disappear more quickly from the tissues of the immunized than of the normal animals. It appears of interest in this connection to inquire into the nature of the factors responsible for this immunity, whether resident in the blood or tissues.

ATTEMPTS TO DETERMINE BY IN VITRO EXPERIMENTS THE FACTORS RESPONSIBLE FOR THE DESTRUCTION OF TYPHOID OR PARA- TYPHOID B. BACILLI IN THE RABBIT BODY

The original conception of Pfeiffer and his pupils, based on experiments primarily conducted on guinea-pigs, attributed the resistance to typhoid fever to bacteriolytic properties of the blood and lymph stream. This interpretation of the acquired immunity was challenged by Metchnikoff and his school, who believed that the leukocytes were mainly responsible for the destruction of the typhoid bacillus in the blood stream or the tissues of the immune animal or man. Based on the well-known experiments of Wassermann and Citron,²⁷ which demonstrated that antibodies may be produced by any cell or cell complex brought in contact with pathogenic bacteria, the theory of the local tissue immunity was proposed, but attracted little attention. However,

²⁷ Deutsch. med. Wchnschr., 1905, 31, p. 573.

in recent years the conception that in man the resistance to typhoid fever acquired during recovery from this disease is cellular and not humoral, has gained some encouraging experimental support. In this connection the studies of Wassermann and Sommerfeld²⁸ on mice, of Besredka²⁹ on rabbits, and of Liebermann and Acel³⁰ on guinea pigs, and the critical summary of Bessau,³¹ which bridges the humoral and cellular theory in an attractive manner, should be mentioned. These studies are of great value from an experimental standpoint, but as we are unable to state whether the immunity mechanism of the guinea-pig, rabbit or mouse is identical with that in man, far reaching conclusions and analogy deductions are unquestionably premature and unjustified.

It is interesting to read in many publications dealing with the behavior of the typhoid bacillus in experimental animals, the statements that it is impossible to provoke in the usual laboratory animals, including the chimpanzees, a disease similar to typhoid fever in man, and that for these reasons the various factors and processes accompanying a human infection cannot be reproduced, nor can they be followed in the course of their intricate development. Yet the same writers usually untrained in comparative pathology, do not hesitate to regard certain facts elucidated, for example, on rabbits as indicative of a close analogy to the infection produced by the typhoid bacillus in man. It appeared to us that progress in the understanding of the mechanism of typhoid resistance could perhaps be made by analyzing our knowledge concerning the nature of the factors responsible for the native and acquired immunity of the rabbit against the typhoid or paratyphoid B bacillus. The *in vivo* experiments reported in the preceding paragraphs confirmed the observations of Bull, Arima, and Parker and Franke. Typhoid bacilli intravenously injected into rabbits are primarily destroyed in the liver, spleen, bonemarrow and lung. Even in the immunized rabbit the progress of the destruction of the deposited bacilli differs in no respect from that established for the normal animal. The test-tube experiments of Buxton,³² Bull,³³ Teague and McWilliams,³⁴ and Stone showed, however, that the blood serum, plasma or defibrinated blood of immunized rabbits is nonbactericidal for the

²⁸ *Med. Klin.*, 1915, 11, p. 1307.

²⁹ *Ann. de l'Inst. Pasteur*, 1919, 33, pp. 301 and 557.

³⁰ *Deutsch. med. Wehnschr.*, 1917, 43, p. 867; 1918, 44, p. 313; *Biochem. Ztschr.*, 1918, 91, p. 46.

³¹ *Deutsch. med. Wehnschr.*, 1916, 42, p. 499.

³² *Jour. Med. Research*, 1905, 13, pp. 305 & 431.

³³ *Jour. Exper. Med.*, 1914, 20, p. 237.

³⁴ *Jour. Immunology*, 1917, 2, pp. 167, 185 and 375.

typhoid bacillus, while normal serum can destroy in 5-24 hours from 100,000-5,000,000 organisms. As numerous experiments conducted by us with the blood, its fluid and cellular elements produced results similar to those reported by these workers, it is considered unnecessary to present our additional data. It therefore may be surmised that the destruction of typhoid bacilli in the normal or unimmunized rabbits either cannot primarily be the result of bacteriolytic serum or lymph substances, or the in vitro experiments fail to reproduce the conditions as they exist in the rabbit body, a view held by Bail in 1905. The latter conception is apparently supported by some tests which we conducted with the serum of rabbits immunized against paratyphoid B bacilli.

Exper. 10. Action of the Serum and Whole Blood of Normal and Immunized Rabbits in Vitro on Paratyphoid B Bacilli (B. Aertryckei).—The defibrinated blood or the fresh serum of normal healthy rabbits, or of those immunized by 3 subcutaneous injections with small doses of living paratyphoid B bacilli were tested for agglutinating value and for bactericidal power by means of the Buxton³² or Teague and McWilliams³⁴ technic.

Thermolabile agglutinins for paratyphoid B bacilli in dilutions varying from 1:2-1:10 were demonstrated in the 4 rabbits tested for this purpose. The immune agglutinins of the protected rabbits reached frequently a titer of 1:10,000 and over.

One cc of fresh serum obtained from the heart blood of the normal and immunized rabbits was absolutely devoid of bactericidal properties for the *B. aertryckei* (rabbit paratyphoid B). Not even 5-15 organisms were destroyed in 24 hours. These serums in equal amounts were readily able to kill from 40,000-500,000 typhoid bacilli after an exposure of 24 hours. Similiar results were obtained with defibrinated blood. Human paratyphoid B bacilli reacted more irregularly; some rabbit serums would destroy from 100-1,000 bacteria. The human strains employed were about 2 years old; they had lost their invasive properties for the rabbit and in turn became more susceptible to the bactericidal powers of the blood.

These observations are not new. Citron,³⁵ Toepfer and Jaffe,³⁶ Neufeld and Hüne,³⁷ Werbitzki,³⁸ and recently Teague and McWilliams³⁴ have noted that the normal or immunized rabbit serum may possess no bacteriolytic substances while tropins can be demonstrated. In fact, Citron³⁵ and Tenbroeck³⁹ concluded from numerous experiments with the *B. suipestifer* that the presence or absence of serum antibodies does not permit conclusions relative to the degree of resistance of the rabbit against a subsequent infection.

³⁵ Ztschr. f. Hyg. u. Infektionskrankh., 1906, 53, p. 515.

³⁶ Ibid., 1905, 52, p. 407.

³⁷ Arb. a. d. k. Gsndhsamte, 1907, 25, p. 164.

³⁸ Arch. f. Hyg., 1909, 70, p. 271.

³⁹ Jour. Exper. Med., 1917, 26, p. 441

The evidence thus far at our disposal suggests, therefore, that the destruction of typhoid as well as paratyphoid bacilli in the immunized rabbit cannot be explained by in vitro experiments with the whole blood or serum. Impressed by the conception of an histogenous immunity, so ably demonstrated for streptococcus infections in the cat, rabbit and guinea-pig (Hopkins and Parker, and Nagao),⁴⁰ and for staphylococcus infection in the dog (Bartlett, Ozaki⁴¹ and Ito⁴²) we were led to conduct numerous in vitro experiments with the tissues of infected normal and immunized rabbits. Typhoid and paratyphoid bacilli were used. One experiment is chosen to demonstrate the results obtained.

Exper. 11. Bactericidal Effect of the Tissues of the Normal and Immunized Rabbit Against B. Typhosus in Vitro and Vivo.—Two normal rabbits and 2 members of the same litter, which had been immunized with living typhoid bacilli according to Bull's method, were inoculated intravenously with 2,250,000 typhoid bacilli strain K. per 100 gm. of body weight. The average weight of the 4 rabbits was 2,860 gm. At stated intervals the animals were bled to death under ether. The organs were removed and distributed in sterile test tubes, which were placed in a water bath at 37 C. for from 2-5 hours. At the end of this time the pieces were weighed, ground and plated, as stated in previous experiments. The bactericidal effect of the leukocytes was tested by using clotted blood. The method given by Hopkins and Parker was followed. The results are summarized in table 9.

It will be noted from table 9 that at the end of the first 2 hours of incubation, the lung tissues of both the normal and immunized rabbits exerted in vitro a distinct bactericidal effect on the typhoid bacilli. There was a similar reduction in the spleen and bonemarrow, while in the liver, mesenteric lymph nodes, and kidneys the organisms multiplied moderately. Destruction by the excised tissue took place only during the first 2 or 3 hours of incubation, after which, owing probably to death of the cells, the organisms multiplied. On the other hand, in vivo the destruction progressed continuously and primarily marked in the lung and liver. At the end of the first 12 hours the lung tissue contained from $\frac{1}{30}$ - $\frac{1}{50}$, and the liver from $\frac{1}{40}$ - $\frac{1}{80}$ of the original number of typhoid bacilli deposited in these tissues. The spleen was less active, while the bonemarrow showed a distinct increase. The data pertaining to the clotted blood suggested that the leukocytes had exerted their supposed bactericidal effect. It should be stated, however, that the method of obtaining these results was not very accurate. In control

⁴⁰ Jour. Infect. Dis., 1920, 27, p. 327.

⁴¹ Jour. Med. Research, 1916, 35, p. 465.

⁴² Ibid., 1917, 37, p. 189.

TABLE 9

BACTERICIDAL EFFECT OF THE TISSUES OF THE NORMAL AND IMMUNIZED RABBIT AGAINST B. TYPHOSUS IN VITRO AND VIVO

Tissues	Rabbit 1287; Immunized and Agglutinated; 1:100,000; 6.3 Billion Injected			Rabbit 1287 (Normal 1:20+); 5.7 Billion Injected			Rabbit 1288 (Immune) (5,040,000,000)		Rabbit 1288B. (Normal) (6.3 Billion)	
	10' after Infection, Plated Immediately*	Plated after 2 Hours' Incubation*	Plated after 5 Hours' Incubation*	10' After Infection, Plated Immediately*	Plated After 2 Hours' Incubation*	Plated After 5 Hours' Incubation*	Exsanguinated and Plated 12 Hours after Injection*	Exsanguinated and Plated 12 Hours after Injection*	Exsanguinated and Plated 12 Hours after Injection*	Exsanguinated and Plated 12 Hours after Injection*
Left and central liver.....	300,000	980,000	2,650,000	900,000	810,000	3,000,000	8,000	8,000	5,000	5,000
Right and central liver.....	4,800	4,800	6,800	6,800
Spleen.....	130,000	110,000	2,800,000	550,000	460,000	7,200,000	59,000	59,000	26,000	26,000
Bone marrow.....	62,000	25,000	800,000	21,000	15,000	22,000	225,000	225,000	88,000	88,000
Mesenteric lymph nodes.....	1,670	1,240	15,000	200	100	2,600	84	84	28	28
Lungs.....	70,000	30,000	700,000	20,000	10,000	60,000	1,200	1,200	740	740
Kidneys.....	1,600	3,600	40,000	2,200	1,800	27,000	112	112	64	64
Urine.....	0	0	0	0	0	0
Femoral muscle.....	10	6	1	1	0	0	2	2
Gallbladder wall.....	5,100	28,000	10	10	6	6
Bile.....	1	0	1	1	0	0
Carotis blood.....	(0.4 c c)	(0.7 c c, also E)	(0.8 c c)	(0.8 c c)	(1.8 c c)	(1.8 c c)
Right ventricle.....	1,200 per c c	5,500	1,500; 2,000;	1,500; 2,000;	840; 760	840; 760
Left ventricle.....	900 per c c	per c c	1,000 per c c	1,000 per c c	per c c	per c c
1 c c of blood clotted imme-	300 per c c	per c c	1,800	1,800	640	640
diately.....	320	50	36	1,400 per c c	per c c	per c c	per c c	per c c
Duodenal contents.....	13 colonies B. typhosus	1,750	140	230; 300
				Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus

* Number of colonies per 100 mg.

experiments a moderate multiplication took place, even in the first 3 hours of incubation. The solution of the question dealing with the typholytic effect of rabbits' leukocytes, in the light of the extensive investigations of Bail, Schneider and others, was sufficiently important to warrant a more detailed inquiry than we were able to conduct, at least for the present.

The degree of the destructive action of lung tissue *in vitro* is not constant. In some experiments it is decidedly more marked than in the one just reported. This statement is supported by the figures in table 10.

TABLE 10
COLONIES PER 100 MG. OF LUNG TISSUE

	Rabbit 1265B Typhoid	Rabbit 1265 Typhoid	Rabbit 1142 Typhoid Immunized	Rabbit 1412 Para- typhoid B Immunized	Rabbit 1481 Para- typhoid B Normal
Plated immediately.....	14,400	110,000	226,000	20,800	3,800
Plated after 4 hours' incubation....	4,900	1,380	26,000	159,000	100,000

Observations made with paratyphoid B infected rabbits demonstrated a complete absence of bactericidal powers of the organs on incubation *in vitro*. Even the lung tissues were inactive. These facts are illustrated by the data presented in tables 8 and 10.

The results of the various experiments apparently established the fact that the organs of rabbits, with the exception perhaps of the lung, are unable to destroy typhoid or paratyphoid bacilli *in vitro*. The liver particularly, which *in vivo* is especially endowed to dispose of enormous numbers of bacteria, is inactive in the test tube. An explanation of this phenomenon is not possible, but it is certain that the bactericidal power of the rabbit organs is not only closely connected with a function of living protoplasm but is probably the result of an interaction between the blood and lymph substances and the tissue elements. Whether the polymorphonuclear leukocytes perform this function either alone or in conjunction with endothelial living cells and endothelial leukocytes (N. C. Foot),⁴³ together with certain blood plasma elements has not been determined. The histologic studies of Kyes,⁴⁴ of Hopkins and Parker,⁴⁵ of Bartlett, Ozaki and Ito⁴¹ and of Nagao⁴⁰ with gram positive organisms, such as pneumococci, streptococci, staphylococci,

⁴³ *Ibid.*, 1919, 40, p. 353.

⁴⁴ *Jour. Infect. Dis.*, 1916, 18, p. 277.

⁴⁵ *Jour. Exper. Med.*, 1918, 27, p. 1.

respectively, and the observations of Schneider ⁴⁶ with typhoid bacilli, indicate that both fixed and wandering phagocytes (macrophages) have the ability to engulf, as well as to digest, micro-organisms. The histologic studies of Mallory ⁴⁷ and Gräff ⁴⁸ established conclusively the fact that the typhoid bacillus produced in the reticulo-endothelial tissues of the lymph nodes, spleen, bonemarrow, liver, etc., a specific proliferation of the tissue histiocytes (Aschoff and Kiyono ⁴⁹). A casual study of the sections derived from the animals used in this report clearly demonstrated the remarkable phagocytic action of the endothelial cells of the liver and spleen and confirmed the well-known active function of the polymorphonuclear leukocytes. Several attempts to study the localization of the typhoid bacilli in their relation to the various cells have not been regularly successful. Even the best staining method (thionine, method of Zieler) produced indistinct and misleading pictures. Occasionally Kupffer's cells were found to be packed with bacilli when rabbits killed on the 6th hour were studied. Attention was therefore given to the general appearance of the various cells. Proliferation of the vascular endothelium in the spleen, liver, lung and lymph nodes of rabbits infected with *B. aertryckei* has already been mentioned by one of us in a previous communication, and it was pointed out on this occasion that these vascular reactions suggested a distinctive cellular mechanism of defense. The changes which can be noted in the sections were remarkably similar to those recently described by Nagao ⁴⁰ for the streptococcus, and a detailed account of the data is contemplated in a paper of this series.

The functions of the polymorphonuclear leukocytes as phagocytes, or as sources of typholytic substances, or as cells endowed with specific immunizing properties (Bachman ⁵⁰) cannot be denied since these properties can be demonstrated in vitro and vivo. The observations of Bull ⁷ on phagocytosis in vivo have been confirmed repeatedly. In thionine-stained smears made from the lung tissue, in preference to the liver and spleen pulp, of rabbits which had been inoculated with moderately large doses of *B. typhosus* and killed 10-60 minutes after the injection, we were able to count numerous polymorphonuclear leukocytes retaining intact and phagolyzed rods. In the paratyphoid B infected animals this phagocytosis occurred to a noticeable degree in the

⁴⁶ Arch. f. Hyg., 1909, 70, p. 41.

⁴⁷ Jour. Exper. Med., 1898, 3, p. 611.

⁴⁸ Deutsch. Arch. f. klin. Med., 1918, 125, p. 352; 126, p. 1.

⁴⁹ Folia haematol., 1913, 15, p. 383.

⁵⁰ Rev. de la Assoc. méd., Argentina, 1918, 29, p. 549; Rev. méd. del Rosario, 1919, 9, p. 1; Prensa méd., Argentina, 1919, 5, p. 253.

immunized, and to a lesser degree, in the normal rabbit. Whether this phagocytosis, however, only initiates the destructive mechanism cannot be investigated with certainty *in vivo*. We gained the impression that it was more or less a temporary process, but the subject will be considered more carefully in the next paper in connection with the analysis of the mechanism leading to the elimination of bacilli in the bile. Opportunity will be afforded to discuss the changes in the distribution of the polymorphonuclear leukocytes, which follow an intravenous injection in the first 5-120 minutes. Since the bacilli are taken up by the leukocytes it appears not at all unlikely that they are digested by a leuko-enzyme; this conception is supported by the demonstration of an ereptic enzyme in the rabbit's leukocyte by Parker and Franke.⁵¹

We desire to recall the observations of R. Schneider,⁵² who showed by a set of ingenious experiments the active secretion of thermostable bactericidal substances by leukocytes suspended in inactivated serum. This vital secretory function of the leukocytes is readily paralyzed by asphyxia of the cells with CO₂. The *in vitro* experiments conducted with rabbits' tissues by us and others have ignored this important fact. Is it not possible that the CO₂ content of the liver, spleen and bone-marrow *in vitro* inhibits the function of the leukocytes? It is, however, possible that the liver and spleen contained few leukocytes at the time the rabbit was exsanguinated, and the lung retaining the majority of these cells was particularly endowed to exhibit the described bactericidal properties. An investigation, with these conceptions in mind, is earnestly needed, as positive results would materially help to elucidate the many unexplainable impressions obtained through test-tube experiments. Attention should also be paid to the recent studies of Bachman⁵⁰ on a specific substance demonstrable in the polymorphonuclear leukocytes of immunized animals. This may, for example, prove that the leukocytes of rabbits immunized against paratyphoid B display a new property even *in vitro*, namely, a specific phagocytosis.

From this discussion it is quite evident that bactericidal substances directed against the typhoid and paratyphoid B bacilli are primarily of cellular origin, and are probably produced when needed at the places of bacterial invasion. In reviewing these facts one is once more reminded of the early studies of Wassermann and Citron⁵³ on local tissue immunity. According to the observations of these workers, it is

⁵¹ Jour. Med. Research, 1919, 39, p. 301.

⁵² Arch. f. Hyg., 1909, 70, p. 41.

⁵³ Deutsch. med. Wchnschr., 1905, 31, p. 573.

proved that the tissues which come in intimate contact with the typhoid bacilli are capable of reacting locally by the production of specific bactericidal substances. For example, the cells lining the pleural cavity cannot only bind the specific antigen, but respond promptly by a production of bactericidal substances. Besredka⁵⁴ expresses a somewhat similar idea in his explanation of the intestinal immunity against paratyphoid-dysentery bacilli in the rabbit: "l'immunité naturelle vis à vis du virus typho-paratyphique, l'immunité acquise artificiellement repose également sur l'immunité de la paroi intestinale, ce qui veut dire qu'elle est locale."

Some observations on the spleen discussed in the preceding paragraphs suggest that such a mobilization of bactericidal substances takes place in the organ itself. Ozaki⁵⁵ demonstrated that the accumulation of bacteria in the spleen was principally dependent on the vital activity of the cells, and Morris and Bullock⁵⁶ inferred from their extensive experiments that the spleen normally aids tremendously in resisting infectious processes in rats. These facts directed our attention to the spleen. It was constantly noted that in animals of the same litter the disappearance of the deposited typhoid bacilli was slow in the first 1-2 hours after the inoculation, irrespective of the marked local hyperleukocytosis and in contrast to the great reduction of their number in the liver and kidney. For example, in the first hour after the introduction of the infective dose, the spleen contained $\frac{1}{3}$ of the originally deposited bacilli, while at the end of the fourth hour, $\frac{1}{12}$; at the fifth, $\frac{1}{25}$; at the eighth, $\frac{1}{80}$; and at the end of the 24th hour, $\frac{1}{250}$ to $\frac{1}{400}$ of the original number was recordable in the spleen by the plating method. The figures dealing with the liver of the same animal were at the 8th hour, $\frac{1}{40}$; and at the end of the 24th hour, $\frac{1}{100}$ of the original number could be noted. These findings are subject to several explanations. From the recent studies of Teague and McWilliams,⁵⁷ it would seem that bacteriolytic substances could readily pass from the blood capillaries to the lymph, and by this route to the spleen and liver, which had been heavily invaded by typhoid bacilli. This hypothesis is applicable to an explanation of the destruction of typhoid bacilli in normal rabbits, but as these forces are not demonstrable in the typhoid and paratyphoid immunized rabbits, the conception of a cellular or a histohumoral immunity mechan-

⁵⁴ Bull. de l'Inst. Pasteur, 1920, 18, p. 129.

⁵⁵ Jour. Med. Research, 1917, 36, p. 413; 37, p. 247.

⁵⁶ Ann. Surg., 1919, 70, p. 513.

⁵⁷ Jour. Immunology, 1917, 2, p. 375.

ism has far more justification. No doubt the suggestions of Manwaring and Coe,⁵⁸ dealing with the so-called endothelial opsonins, and the histologic studies of Nagao,⁴⁰ also deserve some attention in future experimental investigations.

Additional observations illustrating phases of local immunity in the liver of immunized rabbits and guinea-pigs will be presented in paper 5. In connection with these experiments, it was repeatedly noted that the liver of rabbits which rapidly destroyed enormous numbers of typhoid bacilli were exceedingly voluminous, soft and heavy. For example, rabbits weighing from 2,000 to 2,200 gm. possessed, 4 to 8 hours after the inoculation, livers weighing 122 to 128 gm. These organs instead of representing $\frac{1}{30}$ had increased to $\frac{1}{16}$ of the body weight. A marked edematous infiltration characterized such livers histologically, and the condition must be interpreted as lymphostasis. We are not prepared to draw definite conclusions from this observation, but the well-known fact is recalled that rabbits' lymph obtained by stasis is strongly bacteriolytic for typhoid bacilli, and that the typholytic substances of such body fluids are of leukocytic origin (Schneider⁵² and others).

The ability of excised pieces of lung tissue to destroy *in vitro* a large number of typhoid bacilli is apparently a fairly common function, having been demonstrated for streptococci and staphylococci. Moreover, it is certain that the perfused organ, freed from blood and, to a certain degree, of lymph, is capable of this action. Intercurrent infections, such as bronchopneumonia caused by *B. bronchisepticus*, seriously impair this destructive property. Moreover, extracts of the lung tissue are inactive. Whether some of the bactericidal substances (polymorphonuclear leukocytes) are preformed in the native immune rabbit, or whether they are mobilized locally, as in the paratyphoid B susceptible animal, is as yet unknown. According to Deutsch⁵⁹ the lung tissue of normal guinea-pigs always contains more agglutinins than the serum, while Briscoe⁶⁰ claims that phagocytosis by the pulmonary macrophages is more active and rapid when the animal has been previously immunized. The evidence thus far collected experimentally indicates, however, that the bactericidal substances are dependent on the action of the living cells. The entire mechanism of immunity in the lung is probably strictly cellular in origin and action, and is not influenced by a process of

⁵⁸ *Ibid.*, 1916, 1, p. 401.

⁵⁹ *Ann. de l'Inst. Pasteur*, 1899, 13, p. 689.

⁶⁰ *Jour. Path. & Bacteriol.*, 1907-08, 12, p. 66.

immunization. This definitely demonstrable histogenous bactericidal property, which is inherent in the lung is, however, of subordinate influence on the general mechanism of typhoid immunity in the rabbit. A careful experimental analysis of the pulmonary bactericidal powers will no doubt materially assist in disproving that humoral factors alone govern defensive and destructive immunity in typhoid fever and allied diseases.

THE NATURE OF IMMUNITY IN THE RABBIT AGAINST THE TYPHOID AND PARATYPHOID B BACILLUS

The studies of Bail,⁶¹ of Hiss⁶² and of Parker and Franke⁵¹ indicate that the disease artificially produced in rabbits by the intravenous injection of typhoid bacilli is rather a toxemia than an infection ("ein Giftod"—Bail; "acute intoxication"—Hiss). Parker⁶³ has recently proved that the livers of very sick rabbits contain specific saline-extractable poisons. These toxins are of cellular origin and are the product of an interaction between the living cells of the liver, of other organs and the killed and disintegrated typhoid bacteria. Intoxication and death result primarily in those rabbits that possess a marked power to destroy bacteria in the liver and spleen. The susceptibility is therefore due to the bactericidal properties of its blood and tissues. Resistance to this intoxication is produced by active immunization with living typhoid bacilli, while typhoid immune serums failed to protect against the liver poison. We were able to confirm fully the observations of these workers. Rabbits capable of destroying in 1 c c of serum from 1,000,000 to 5,000,000 typhoid bacilli readily succumbed to the intoxication in from 5 to 48 hours, when injected with from 5 to 10 billion typhoid bacilli; and extractable liver poisons could be demonstrated in every instance. On the other hand, rabbits lacking typholytic substances in the blood or serum, and particularly immunized animals, would show symptoms of intoxication, although liver poison could not be found. The nonsusceptibility is either due to an absence of lytic antibodies or the result of a tolerance to the liver poison acquired in the course of the process of immunization. On the other hand, rabbits injected with paratyphoid B bacilli in moderate doses (less than 1,000 million) fail to show signs of intoxication for at least 48 hours; at the time of death their blood and organs contain enormous numbers of

⁶¹ Arch. f. Hyg., 1905, 52, p. 272; Wien. klin. Wchnschr., 1907, 20, p. 275.

⁶² Jour. Med. Research, 1913, 28, p. 389.

⁶³ Jour. Exper. Med., 1918, 28, p. 571.

bacteria, but no poisons can be extracted from the liver. Paratyphoid infections in the susceptible rabbit exhibit all the signs of a septicemia. Instead of a progressive destruction of organisms in the blood and organs, as is shown for the typhoid rabbit, a progressive increase takes place. Resistance to this group of bacteria is developed by factors which prevent their multiplication. In vitro experiments indicate that this property is not in the blood serum, but confined to the living cells, and is only acquired by active immunization with living paratyphoid bacilli, a fact demonstrated by Citron³⁵ and TenBroeck.³⁹ The invasive *B. aertryckei* or the *B. suispestifer* produces a septicemia in the rabbit, while the typhoid bacillus causes a toxemia. It should, however, be borne in mind that only very large doses of living typhoid bacilli are capable of causing this intoxication in susceptible rabbits, while even small doses of paratyphoid B bacilli produce a fatal septicemia. Moreover, the defensive mechanism against the intoxication, which saves the life of the animal, is called on during the first 10 hours after the injection of the typhoid bacillus, while in the paratyphoid infection a similar mechanism may play a rôle only toward the end of the disease. It is, however, well known that typhoid rabbits may die from a moderately large initial injection on the 3rd to the 10th day. In these instances we have failed to find extractable liver poisons, but have demonstrated enormous numbers of bacteria in the bonemarrow, a moderate number in the spleen and liver, and few or no organisms in the blood. For illustration of this point the reader is referred to rabbit 1686 in table 6. Our observations unquestionably corroborate the conclusions of Parker and Franke that in rabbits which are slowly dying of typhoid the bonemarrow may constitute a focus of infection from which the organisms are continually swept into the blood stream and from there taken up by the organs. The actual mode of this localization, whether in the epiphysis marrow or elsewhere, is of great interest but by no means clearly understood (I. Koch⁶⁴). Comparative leukocyte counts by Studer,⁶⁵ C. W. Wells⁶⁶ and our own indicate that the individual response of the bone-marrow of rabbits, following the injection of typhoid bacilli may vary considerably. In a certain percentage of animals serious disturbances in the leukocytogenic functions follow the injection of typhoid, paratyphoid and coli bacilli, which in turn lead to an exhaustion of the leukocytes and to a breakdown of the defensive

⁶⁴ Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 436.

⁶⁵ Thesis, Univ. of Zürich, 1903.

⁶⁶ Jour. Infect. Dis., 1917, 20, p. 219; 1918, 22, p. 502.

mechanism of the body. Future studies should attempt to correlate these observations. It is, however, not unlikely that the numerous infarcts of the liver and spleen going on to necroses as the result of typhoid toxemia constitute also seed beds for the invasion of the blood stream. These toxic necroses lack proper lymph circulation, according to Gräff⁴⁸ and favor, as has been demonstrated by Benian,⁶⁷ the development of immune body-fast, parasitic typhoid bacilli. Furthermore, the protection of pathogenic organisms by living, endothelial cells, shown by Rous and Jones,⁶⁸ must be kept in mind. Unhindered development of the bacteria in the gallbladder constitutes, in our experience, only in exceptional instances a focus responsible for the infection of the blood stream, as will be demonstrated in paper 5, on the mechanism of gallbladder infection.

Continuous invasion of the blood stream and removal of the organisms by the viscera is accompanied, in the first place, by an accumulation of typhoid bacilli in the liver and in the spleen. In these tissues the main destruction takes place; the liberation of toxic albumoses by disintegration, or the production of toxic substances by some form of proteolysis, unquestionably produces a profound injury, which can be seen macroscopically. The inability to demonstrate poison in the liver is probably due to the fact that its liberation is gradual and in amounts which do not exceed the detoxicating threshold of the organs. Yet this detoxicating function, which probably is present in other organs than the liver, continues as long as bacteria are swept into the blood from the various foci mentioned; but the prolonged production of toxins may seriously damage the metabolic function of the organs, causing cachexia and the death of the animal. Immunized rabbits, which succumb to very large injections of typhoid bacilli, present the same cultural findings (of a low blood and a high bonemarrow count). It appears, therefore, not unlikely that aside from the tolerance against specific poisons, immunity against the typhoid bacillus in rabbits is represented by their ability to prevent the formation of bonemarrow and visceral foci.

In rabbit paratyphoid the distribution of bacteria in the blood and organs is quite uniform shortly before death on the 6th or 7th day, irrespective of the original route of infection. The blood contains large numbers of bacteria per c c, and the tissues a proportional share, according to size. The septicemia is probably the consequence of the washing

⁶⁷ Jour. Path. & Bacteriol., 1920, 23, p. 171.

⁶⁸ Jour. Exper. Med., 1916, 23, p. 601.

out of organisms from the foci of active multiplication. The enlarged spleen, in case the gallbladder is not infected, is the chief source; while the bonemarrow, liver and lungs are less important. Liver poisons cannot be demonstrated for the reasons above outlined, but the anatomic lesions and the clinical symptoms suggest that the death of the animal is probably the result of an intoxication. Immunity against a paratyphoid B infection consists, therefore, in an ability to prevent the invasion and multiplication of the bacteria, and with it the development of poisons which injure the function of the important organs. This immunity mechanism is effective following the subcutaneous inoculation of living bacteria, but it can be readily overcome by the intravenous inoculation of moderately large doses of paratyphoid B bacilli. Overwhelming blood infections of the immunized rabbit produce a cycle of events identical to that described for the typhoid animals. The intoxications provoked by the inoculation of heat-killed paratyphoid bacilli or their extracts have not been investigated, and therefore no attempt is made to explain the mechanism of susceptibility and nonsusceptibility to preformed toxins.

From this discussion it is evident that the disease produced in the rabbit by intravenous injection of typhoid or paratyphoid B bacilli must be considered a specific toxicosis. In this term we include the rôle played by so-called endotoxins, and assume in addition that toxic substances (perhaps amines) are produced from the host's protein by some form of proteolysis, which is stimulated by the disintegrated bacteria and their products. The abrupt increase in the nitrogenous excretion (primarily the allantoin fraction) of rabbits injected with living typhoid bacilli, demonstrated by Pepper and Miller,⁶⁹ is no doubt a distinct indication of an extensive katabolism of body protein. Resistance to this form of experimental disease is a local or general "antitoxic adaptation immunity" and cellular in nature. The native immunity of rabbits for the typhoid bacillus depends entirely on (1) the ability of the endothelial cells, polymorphonuclear leukocytes, and perhaps the body fluid to destroy the invading bacteria; (2) the mechanism to prevent the formation of foci from which the blood stream is constantly seeded; and (3) the detoxicating power of the liver and the tissues in general. These factors, inherent in the rabbit, can be made inoperative by the injection of a very large dose (over 5,000 million) of typhoid bacilli. An acquired immunity by means of dead or living vaccines can even then prevent the

⁶⁹ Jour. Infect. Dis., 1916, 19, p. 694.

final issue produced by the exhaustion of the bonemarrow with its resulting toxemia. Such an acquired resistance is primarily cellular and mainly directed against the liberated toxins. It appears that the actual destruction of the bacteria and the elimination of foci responsible for the continuous invasion of the blood stream is managed by the same, perhaps slightly enhanced, cellular factors responsible for the inherent native immunity.

Susceptibility of the rabbit to paratyphoid B bacilli resolved itself into an inability of certain tissues and their cells, perhaps together with the blood, to kill the introduced bacteria completely. As a result, foci occur in the least resistant tissues (bonemarrow and lymphatic tissues), and the blood stream and organs are gradually invaded with newly developed bacteria. Organs like the liver and spleen, which were able to free themselves to a large extent of living paratyphoid bacilli during the first period of the infection, are then overwhelmed with bacteria and their toxins. Immunity produced by recovery from a slight infection consists of a fairly speedy sterilization of the tissues and blood, together with the elimination of foci, which act as seed beds for the ensuing septicemia in susceptible animals. This is probably not the same mechanism as that evolved by immunization with dead bacteria.

Various questions suggest themselves; for example, Is the immunity produced in rabbits against typhoid or paratyphoid bacilli comparable with the one provoked in man by the prophylactic inoculation of vaccines? From the information at hand it is impossible to answer this important question. Our experiments on guinea-pigs, cats, and monkeys indicate that each species disposes of the intravenously injected organisms of the typhoid-paratyphoid group in its own characteristic way. The immunized guinea-pig destroys typhoid and paratyphoid bacilli more readily than the normal; while monkeys and cats behave like the rabbit. It is quite possible that typhoid fever in man really is a toxico-sis, or an endotoxin disease, as proposed by Stadelmann and Wolf-Eisner.⁷⁰ The immunity mechanism could then follow either the course outlined for the typhoid or the paratyphoid B infection in the rabbit. It may, however, be entirely different and be more readily comparable with the conditions found in the guinea-pig. The available bacteriologic and immunologic data pertaining to typhoid fever in man are not sufficiently complete to venture these far-reaching deductions based on analogy.

⁷⁰ München med. Wehnschr., 1907, 54, pp. 1161 and 1237.

CONCLUSIONS

A small number of typhoid bacilli inoculated intravenously into normal rabbits of the same litter was rapidly removed from the blood stream at the end of from 10 to 15 minutes after the inoculation. About 20 to 30% of the inoculum were found in the liver; a smaller amount in the spleen, bonemarrow and lungs; and comparatively small numbers in the lymph nodes, kidneys, muscle, etc.

The gallbladder bile contained only a small number of bacteria, while the gallbladder wall received, according to its size, a proportional share of the total number of bacteria deposited in the liver. The intraportal injection presented no advantage over the common intravenous method.

Large numbers of typhoid bacilli are less speedily removed from the blood stream, but the distribution in the organs remains the same in the first 4 to 8 hours.

The bacteria taken up by the tissues are rapidly reduced in number, particularly in the liver, spleen and lungs. The bonemarrow is less active and not infrequently the typhoid bacilli multiply in these tissues and form foci, which reinfect the blood stream. The gallbladder can become infected either as a result of an immediate extensive elimination of the introduced bacteria, or through the continuous discharge of bacilli from liver foci in the hepatic bile, or in consequence of an embolic infarction of the gallbladder capillaries causing a diphtheritic cholecystitis. In the latter instance the cystic bile may not become invaded with typhoid bacilli for 24-48 hours after the intravenous injection.

The distribution and destruction of small or large doses of typhoid bacilli is practically the same in the normal as in the immunized rabbit, whether protected by the injection of dead or living organisms. The only noteworthy difference between the normal and immunized rabbit is found in the lung and spleen, namely, the lung of the immunized rabbit takes up a somewhat greater number of bacteria than the normal; and the spleen of the immune sterilizes itself more slowly than the normal.

Localization in the gallbladder and bile 96 to 120 hours after the infection occurs more readily with moderately heavy suspensions prepared from rabbit-blood agar than those obtained from peptic-digest agar slants.

Rabbits which succumb to the typhoid intoxication regularly harbor enormous numbers of typhoid bacilli in the bonemarrow of the long

bones; these foci are probably the seed beds responsible for the continuous invasion of the blood stream and the subsequent overwhelming infection of the liver and spleen.

Paratyphoid A bacilli act in a manner identical to typhoid bacilli. The mechanism of destruction of paratyphoid B bacilli, whether *B. aertryckei* of rabbit origin, or *B. schottmülleri* in the immunized and normal rabbit, is different from that reported in the foregoing for the typhoid bacillus. As a rule, paratyphoid bacilli disappear more quickly from the blood and tissues of immunized than normal animals. The activity of the splenic tissue in the destruction of these bacteria is noteworthy and deserves further investigation.

The blood and serum of normal rabbits in vitro is bacteriolytic for typhoid and paratyphoid A bacilli, but is inactive for paratyphoid B bacilli. The serum of immunized rabbits in vitro, whether protected against typhoid, paratyphoid A or paratyphoid B bacilli, is nonbactericidal.

Pieces of excised organs of exsanguinated rabbits heavily infected with bacilli of the typhoid-paratyphoid group when incubated in test tubes at 37 C. may show in the first 2 hours a slight bactericidal action. The property is marked in the excised lung tissues, but not in its extracts and is dependent on the living protoplasm of the cell.

The nature of the immunity of the rabbit against the typhoid and paratyphoid B bacilli is considered in some detail, and it is pointed out that an intravenous injection of a large dose of *B. typhosus* produces primarily a toxemia, while the invasive *B. aertryckei* provokes in small amounts a progressive septicemia. The various cellular factors, which, in the light of available knowledge, are responsible in the normal and immunized rabbit for the local and general antitoxic adaptation immunity are discussed.

THE MECHANISM OF GALLBLADDER INFECTIONS IN LABORATORY ANIMALS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. V

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Experimental gallbladder infections have been produced in the rabbit, guinea-pig, etc., by numerous investigators, following intravenous injections of typhoid, paratyphoid, dysentery and coli bacilli, streptococci, staphylococci and even nonpathogenic organisms. A perusal of the numerous publications indicates that these infections of the biliary system are, however, not developed with any degree of certainty. In spite of the excellent experimental studies of Doerr,¹ Blumenthal,² Else,³ Nichols⁴ and others, our knowledge is still incomplete concerning the exact mechanism which provokes in the rabbit a localization of the inoculated typhoid bacilli. Moreover, it has been customary to look on the gallbladder as the only great breeding place of typhoid bacilli, whenever they have been found in this viscus a few weeks after the inoculation of the infective dose. No doubt there is a considerable accumulation of data pointing in this direction, but there are observations on rabbits and guinea-pigs, namely, those of Hailer and his associates,⁵ and Emmerich and Wagner,⁶ which do not conform to this conception of the carrier state. These workers have demonstrated that in about 10% of instances the cystic bile may be found sterile, although the wall contains typhoid bacilli. The biliary passages may likewise be sterile and yet the liver (in 80% of the instances), the bone-marrow (in 25 to 50% of the instances) and the spleen (in 38% of the instances) may harbor the bacilli for more than 30 days. Moreover, the majority of experimental pathologists who study the gallbladder carrier state in rabbits, overlook the early observation of

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¹ Centralbl. f. Bakteriolog., I., 1905, 39, p. 624.

² Ibid., 1910, 55, p. 341.

³ Surg., Gynec. & Obst., 1910, 11, p. 470.

⁴ Jour. Exper. Med., 1914, 20, p. 573; 1916, 24, p. 497; 1917, 68, p. 958.

⁵ Deutsch. med. Wchnschr., 1912, 38, p. 2267; Arb. a. d. k. Gsndhtsamte, 1914, 47, pp. 303, 451, and 470. Hailer, E., and Wolf, G.: Arb. a. d. k. Gsndhtsamte, 1914-15, 48, p. 80.

⁶ Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

Blachstein;⁷ namely, in chronic or subacute infections of the gallbladder contents the lesions from which the infection occurs exist in the liver in the form of necrotic or inflammatory foci. Keeping these facts in mind, it seems advisable to regard the biliary tract as an excretory channel and the cholecystitis more as a sequel than as the source of the continual and repeated infection of the bile. A similar but novel view has recently been advanced by Webb-Johnson⁸ to explain the human intestinal carrier state. This writer assumes the spleen to be the primary focus responsible for the elimination of typhoid bacilli in the bile. Opportunity will be afforded to discuss these conceptions in the course of the analysis of our experimental data.

Following an intravenous injection of typhoid bacilli, such organisms can be demonstrated quite regularly in the gallbladder, but the duration of their sojourn is exceedingly variable and in many instances limited to a short period. A study of the pathogenesis of the experimental carrier state in laboratory animals must therefore consider (a) the mechanism by which the bacilli reach the gallbladder and (b) the concomitant factors in the form of anatomic or physiologic changes, which either lead to a prolonged persistence or to a rapid disappearance of the invading typhoid bacilli. Among these changes may be mentioned: the pathologic processes in the gallbladder wall (cholecystitis and cholangitis); peculiarities in the physiology of the bile with reference to its secretion, composition and reaction; and abnormalities in the biliary system leading to the formation of gallstones. Consideration must also be given to the question of the individual immunity of the infected animal. It is the purpose of this paper to investigate these contributory factors in connection with their bearing on the mechanism of gallbladder infections.

GENERAL CONSIDERATIONS

It is generally stated that theoretically bacteria may reach the gallbladder in the following manner: (a) direct infection (puncture); (b) ascending infection from the intestines via ductus choledochus; (c) descending infection (with the bile secreted from the liver); (d) arterial embolus of the wall vessels; (e) through the lymphatic and venous blood vessels. Each of these possibilities has been investigated experimentally and has been shown to occur in typhoid cholecystitis in

⁷ Bull. Johns Hopkins Hosp., 1891, 2, pp. 96 and 121.

⁸ The Lancet, 1917, 2, p. 813; Surgical Aspects of Typhoid and Paratyphoid Fevers, London, 1919, p. 167.

the rabbit. Some of the observations have even *sine conditione* been applied to explain human cholecystitis, and the intestinal carrier state. A brief consideration is therefore justifiable to determine, if possible, which of these routes is the one usually followed in the experimental reproduction of gallbladder infection in animals and to what extent the findings are analogous to those present in the human carrier state.

Infection through trauma (puncture) occurs in rare instances only as a result of surgical procedures or manipulations. The direct inoculation of various bacteria into the gallbladder of rabbits and guinea-pigs has been practiced experimentally by Gilbert, and Dominici⁹ and Fournier,¹⁰ Talma, Richardson,¹¹ Italia,¹² Ehret and Stolz,¹³ Forster,¹⁴ Violle,¹⁵ Marxer,¹⁶ Uhlenhuth and Messerschmidt,¹⁷ Klinkert,¹⁸ Hailer and Ungermann,¹⁹ Lange and Roos,²⁰ Schöbl,²¹ Emmerich and Wagner,²² and Venema.²³ The published reports indicate that this method produces with greater regularity a localization of the bacteria in the gallbladder than the intravenous method of infection. Constant results with the typhoid bacillus can be expected in rabbits, according to Hailer and Ungermann, only until the 30th day after the operation. In a few animals the bacilli disappear at the end of this period, while in others a chronic inflammation favors their persistence for from 217 (Hailer and Rimpau) to 341 days (Emmerich and Wagner). The results which are obtained with the typhoid bacillus in guinea-pigs are less satisfactory (Marmoreck.²⁴ Wagner and Emmerich,²⁵ Thompson and Meyer²⁶). The gallbladder infections

⁹ Bull. d. l. Soc. de biol., 1893, 5, p. 1033.

¹⁰ Compt. rend. Soc. de biol., 1897, 49, pp. 692 and 636.

¹¹ Boston Soc. Med. Sc., 1898-99, 3, p. 79.

¹² Policlin., Roma, 1901, 8.—C., p. 153.

¹³ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 1900, 7, p. 372; and 1901, 8, p. 153.

¹⁴ Forster, in Uhlenhuth, P., and Messerschmidt, T.: Deutsch. med. Wehnschr., 1912, 51, p. 2367.

¹⁵ Ann. de l'Inst. Pasteur, 1908, 22, p. 341.

¹⁶ Ztschr. f. Chemotherapie., 1914, 2, p. 23.

¹⁷ Deutsch. med. Wehnschr., 1912, 51, p. 2367.

¹⁸ Berl. klin. Wehnschr., 1911, 48, p. 335.

¹⁹ Deutsch. med. Wehnschr., 1912, 38, p. 2267; Arb. a. d. k. Gsmdhsamte, 1914, 47, pp. 303, 451 and 470.

²⁰ Ibid., 1915, 50, p. 57.

²¹ Jour. Infect. Dis., 1916, 18, p. 307; 19, p. 145; Philippine Jour. Trop. Med., 1916, 11, p. 153; 1917, 12, p. 23.

²² Med. Klin., 1916, 12, p. 74; Zschr. f. Immunitätsforsch. u. exper. Therapie, 1916, 24, p. 557; Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

²³ Berl. klin. Wehnschr., 1917, 54, p. 815.

²⁴ Med. Klin., 1916, 12, p. 275.

²⁵ Centralbl. f. Bakteriologie, 1916, 79, p. 1.

²⁶ To be published.

provoked in rabbits by intracystic infections of cholera vibrios or dysentery bacilli (Schöbl,²¹ Nichols,²⁷ Meyer and Stickel²⁸) are characterized by a benign process, which shows a tendency to rapid recovery. The organisms disappear from the injured viscus at the end of from the 15th to the 30th day. All authors, however, agree that direct inoculation of the gallbladder is the route par excellence for the production of experimental gallbladder infections.

It is obvious that the foregoing method does not explain the route by which the typhoid bacillus reaches the gallbladder in the course of the disease. The early finding of typhoid bacilli in the gallbladder of fatal typhoid cases by Gilbert and Girode, Chiari,³⁰ Pratt, and others, in view of the conception (prevailing at the time of their observations) that typhoid was a purely intestinal disease, was explained by the assumption that the bacillus reached the gallbladder by an ascending route from the intestines through the ductus choledochus. Unquestionably the ingenious experiments of Homén,³¹ Ehret and Stolz, Netter, Naunyn, Doerr, Blumenthal and others, which demonstrated the occurrence of bacteria in the bile subsequent to the ligation of the common duct, contributed greatly to the abandonment of this theory as it pertains to the typhoid bacillus. We have been unable, in a number of experiments, to produce gallbladder infections by the introduction of enormous doses of typhoid bacilli into the duodenum close to the papilla of Vater. These results are analogous to those of Hailer and Ungermann. It is needless to state that these findings apply only to the typhoid bacillus, because in the light of numerous clinical and pathologic observations it seems reasonable to suspect an occasional invasion of bacteria or protozoa by the ascending route. Such a mode of infection is particularly favored by intestinal or biliary stasis. The invasion probably never occurs through the common duct against the bile current except in infections with the entameba, but follows the lymphatics of the duct, as has been definitely demonstrated in ascending renal infections; *B. coli*, *B. dysenteriae* and *Cholera vibrio* infections may be mentioned as possible examples of this type of gallbladder infection. In fact, Schöbl³² has demonstrated experimentally in some guinea-pigs

²⁷ Jour. Exper. Med., 1916, 24, p. 497.

²⁸ To be published.

³⁰ Verhandl. d. Deutsch. path. Gesellschaft, 1907; Ergänzt. heft. z. Centralbl. f. allg. Path. u. path. Anat., 1908, 18, p. 143.

³¹ Ibid., 1894, 5, p. 825.

³² J. Infect. Dis., 1916, 18, p. 307.

the presence of cholera vibrios in the bile 7 to 14 days after the feeding of the bacteria.

Descending or so-called hemato-hepatogeneous infection of the bile has been repeatedly verified experimentally and is now generally accepted. Birch-Hirschfeld³³ was the first to state that micro-organisms reach the gallbladder through the circulation either by way of the entero-hepatic blood stream (vena portae) or the arteria hepatica. It is a proved fact that various micro-organisms inoculated intravenously or into a radical of the portal vein of rabbits, dogs, guinea-pigs, and other animals appear in from 2 to 50 minutes in the bile collected from temporary common duct or permanent gallbladder fistulas or from the cystis-bile at necropsy. The early studies of v. Fütterer³⁴ in 1888-1899 on fistular animals have been verified by Biedl and Kraus³⁵ and by Nichols.²⁷ Pernice and Scagliosi,³⁶ Sherrington,³⁷ Pawlowsky,³⁸ Métin,³⁹ Heck⁴⁰ and others have inoculated animals subcutaneously, intraperitoneally, or intravenously with various organisms and have tested after varying time intervals the bile or urine for the presence of the inoculated bacteria. The results have been irregular. For example, Sherrington reports that after the injection of anthrax bacilli, staphylococci and pyocyanus only 18 biles of a series of 49 proved to be infected, while Heck and Métin record negative results. Similarly disappointing have been the experiments of Carmichael⁴¹ and Else,³ who introduced the bacteria by way of the portal vein. On the other hand, it is stated by Breton, Bruyant and Mezie,⁴² that even the introduction of *B. prodigiosus* by gavage produces in a guinea-pig with a ligated common duct an invasion of the gallbladder bile 3 to 4 hours after the introduction of the bacteria. In our feeding experiments with typhoid bacilli, we have cultivated the bacilli occasionally from the liver, but never from the bile. Even in the course of a successful series of paratyphoid feeding infections, we have not succeeded in demonstrating the bacteria in the bile, although as a rule they have

³³ Lehrbuch d. path. Anat., Ed. 4, 1895, 2, p. 694.

³⁴ Berl. klin. Wehnschr., 1899, 36, p. 58; Medicine, 1895, 1, p. 279.

³⁵ Ztschr. f. Hyg. u. Infektionskrankh., 1897, 26, p. 353; and Zentralbl. f. inn. Med., 1896, 17, p. 737.

³⁶ Deutsch. med. Wehnschr., 1892, 18, p. 761.

³⁷ Jour. Path. and Bacteriol., 1892-93, 1, p. 259.

³⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1900, 33, p. 261.

³⁹ Ann. d. l'Inst. Pasteur, 1900, 14, p. 415.

⁴⁰ Ztschr. f. Hyg. u. Infektionskr., 1907, 56, p. 1.

⁴¹ Jour. Path. and Bacteriol., 1903, 8, p. 276.

⁴² Comp. rend. Soc. de biol., 1912, 72, p. 13.

been abundant in the liver (Litch and Meyer⁴³). This definitely proves that the presence of micro-organisms in the liver is not necessarily followed by an invasion of the bile.

Since Blachstein⁷ found that rabbits, which have been given intravenous injections of atoxic strains of *B. coli* or *B. typhosus*, yield cultures from the bile for many days or weeks subsequently, numerous workers have conducted similar experiments. The observations on rabbits by Miyake,⁴⁴ Adrian,⁴⁵ Doerr,¹ Forster,⁴⁶ Lemi  re and Abraham,⁴⁷ Chiarolanza,⁴⁸ Conradi,⁴⁹ E. Blumenthal,² Morgan,⁵⁰ Tanabe and Takeuchi,⁵¹ Arima,⁵² Bully,⁵³ Perussia,⁵⁴ Johnston,⁵⁵ Hailer and Rimpau,⁵ Gay and Claypole,⁵⁶ Nichols,⁵⁷ Weinfurter,⁵⁸ Gibson,⁵⁹ Lentz, Hailer and Wolf,⁶⁰ Besredka,⁶¹ Flu,⁶² and others with typhoid-paratyphoid and dysentery bacilli; Baroni and Ceaparu,⁶³ Cano,⁶⁴ Sch  bl,²¹ and Creig,⁶⁵ with *Vibrio cholerae*; J. Koch⁶⁶ with staphylococci and streptococci; and Rosenow,⁶⁷ Oph  ls and Smith,⁶⁸ with streptococci, indicate that intravenously inoculated bacteria must reach the gallbladder quite frequently and produce a distinct cholecystitis. It even has been demonstrated by Fr  nkel and Much,⁶⁹ and by

⁴³ Jour. Infect. Dis., 1921, 28, p. 27.

⁴⁴ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 479.

⁴⁵ Ibid., 1901, 7, p. 407.

⁴⁶ M  nchen. med. Wchnschr., 1905, 52, p. 1473.

⁴⁷ Compt. rend. Soc. de biol., 1907, 63, p. 252.

⁴⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1909, 62, p. 11.

⁴⁹ Ztschr. f. Immunit  tsforsch. u. exper. Therap., 1910, 7, p. 158.

⁵⁰ Jour. Hygiene, 1911, 11, p. 202.

⁵¹ Mitt. a. d. Mediz. Gesellsch. z. Osaka, 1910, 9, refer.; Centralbl. f. Bakteriologie, I, Ref., 1911, 50, p. 294.

⁵² Arch. f. Hyg., 1911, 73, p. 265; Centralbl. f. Bakteriologie, I, 1912, 63, p. 424.

⁵³ Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 29.

⁵⁴ Pathologica, 1912, 4, p. 141.

⁵⁵ Jour. Med. Res., 1917, 37, p. 189.

⁵⁶ Arch. Int. Med., 1913, 12, p. 616.

⁵⁷ Jour. Exper. Med., 1914, 20, p. 573, 1916, 24, p. 497.

⁵⁸ Centralbl. f. Bakteriologie, 1914-15, 75, p. 379.

⁵⁹ Jour. Roy. Army Med. Corps, London, 1917, 29, p. 601.

⁶⁰ Arb. a. d. Reichs Gesundheitsamte, 1918, 51, p. 1.

⁶¹ Ann. de l'Inst. Pasteur, 1919, 33, pp. 557 and 301.

⁶² Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.

⁶³ Compt. rend. Soc. de biol., 1912, 72, p. 894.

⁶⁴ Centralbl. f. Bakteriologie, 1913, 72, p. 124.

⁶⁵ Indian Jour. Med. Res., 1913-14, 1, pp. 44 and 59; 1914-15, 2, pp. 1, 28, and 907; 1915-16, 3, pp. 259 and 397; 1917, 4, pp. 651 and 658; 1917-18, 5, pp. 81 and 89.

⁶⁶ Ztschr. f. Hyg. u. Infektionskrankh., 1908, 60, p. 335; 1908-09, 62, p. 1; 1911, 69, p. 436.

⁶⁷ Jour. Infect. Dis., 1916, 14, p. 527.

⁶⁸ Proc. Soc. Exper. Biol. and Med., 1918, 15, p. 113.

⁶⁹ Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 342.

E. Fränkel,⁷⁰ that paratyphoid B or A bacilli may exhibit selective properties whereby they tend to realize in the gallbladder and bile of animals infected by intraperitoneal injections or by feeding. Most of the workers mentioned agree that the bacteria reach the gallbladder in the bile secreted by the liver. In some instances (F. Blumenthal and Nichols) they may arrive in from 5 to 10 minutes. The bile capillaries apparently receive the organisms from the blood of the interlobular veins, the endothelial lining being the only barrier which separates the biliary system from the blood stream. The following questions naturally arise: 1. Is this intrahepatic passage of micro-organisms from the blood to the bile a normal secretory function? 2. Is it a mechanical process? 3. Does this elimination follow the pathologic changes which have been produced in the blood vessel wall by the bacterial toxins? Neither Sherrington nor J. Koch, both careful workers, believe in a true physiologic elimination, but they agree that a transit of bacteria across the hepatic membranes can occur without the detectable presence of blood in the infected bile. It is furthermore stated by Sherrington that the membrane may remain normal, unruptured and impervious to blood cells, while the soluble toxin of the bacteria may injure the barrier sufficiently to cause a slight inflammation which is followed by the passage of the accumulated micro-organisms. His view is well supported by the following facts: First, in his experiments nonpathogenic bacteria never appeared in the bile. Second, J. Koch,⁷¹ failed to produce cholecystitis with avirulent skin staphylococci, injected in small doses. Furthermore, Wyssokowitch,⁷² and also Blachstein considered the liver necroses prerequisites for the infection of the biliary secretions. It has been pointed out in the preceding paper (IV), confirming the observations of Weinfurter and Nichols, that the intravenous injection of typhoid bacilli produces bile invasion or infection only when large doses of recently isolated strains are employed. Dosage, virulence and lesions and not the secretory, detoxifying activity of the liver, are the prerequisites for the passage of bacteria from the blood to the bile capillaries. In the light of these established facts the conception of a purely mechanical passage deserves little consideration. It is most unlikely that the masses of bacteria, which are thrown into the liver capillaries, cause "permeability of the liver filter," by rupturing the

⁷⁰ München. med. Wehnschr., 1918, 65, p. 413.

⁷¹ Ztschr. f. Hyg. u. Infektionskrankh., 1908, 60, p. 335.

⁷² Ibid., 1886, 1, p. 3.

capillary wall and by being flushed with the liver secretion into the biliary capillaries. Our experiments, to be reported later, indicate that the transit of bacteria from the blood to the bile is governed by the endothelial lining of the blood capillaries.

The analysis of the various routes by which bacteria reach the gallbladder does not exhaust all the possibilities. Particularly in connection with the typhoid gallbladder problem, there has arisen a controversy of considerable interest. The descending hemato-hepatogenous passage of typhoid bacilli, as described in the foregoing, was accepted as the most likely mode of bile invasion until J. Koch and Chiarolanza⁴⁸ claimed to have proved that the bile may also become infected through the capillaries of the gallbladder wall. Koch drew his conclusion from the histologic picture in a human case of typhoid cholecystitis, in which he found nests of typhoid bacilli lying in close relation to the capillaries of the folds of the mucous membrane. Lange and Roos,²⁰ Creig,⁷³ and others (Posselt⁷⁴) have more or less accepted this view. At Koch's suggestion Chiarolanza tied the cystic duct of rabbits in two places, and injected immediately following the operation typhoid bacilli intravenously. He recovered them from the gallbladder 24 to 48 hours later. These experiments have been justly criticized by E. Blumenthal and Nichols. A careful scrutiny of the protocols published by Chiarolanza indicates that the bile was bloody even in animals examined in less than 24 hours. Technically, the experiments have been poorly executed; he admits that in the majority of incidences the entire gallbladder has been necrotic. The illustration in Figs. 6 and 7 of his article, which show bacterial emboli in the wall, supply ample proof for this statement. In ligating the cystic duct the accompanying cystic artery was in all probability also tied and hemorrhagic infarction occurred from incomplete collateral circulation.

Those who attempt to refute the general conception of an embolic wall infection, depend for their argument on certain experiments conducted by R. Doerr.¹ This worker also tied the cystic duct, but waited from 3 to 5 days before giving the intravenous injection. Under these conditions no infection occurred. The conclusions drawn from these experiments are for the following reasons invalidated: First, no cultures were made of the gallbladder wall in which a focus of infection may be present from 24 to 72 hours before the bacteria break

⁷³ Indian Jour. Med. Res., 1914-15, 2, pp. 1, 28 and 907.

⁷⁴ Ergebn. d. allg. Path. u. path. Anat., 1919, 19, pp. 351 and 471.

through the epithelium leading to contamination of the cystic bile. Second, only one experiment is reported, and in this no mention is made of the number of animals in which either one-half or one loopful of typhoid culture produced a gallbladder infection. The impression is gained that for the successful experiments at least two loopfuls of culture were injected. In the light of these inconsistencies we feel that the conception of an arterial invasion of the gallbladder in rabbits inoculated with large doses of typhoid bacilli is neither proved nor disproved. Experiments and histologic studies to be reported in this paper make it certain that in addition to the common route, namely, the hemato-hepatogenous one, the transverse route via capillaries of the gallbladder wall may be responsible for the development of an experimental cholecystitis. Observations collected from nearly 500 necropsy examinations on rabbits, the results of which are recorded in paper IV in connection with the immunity experiments, and some cases of spontaneous rabbit paratyphoid have materially strengthened this conclusion. Moreover, it has already been proved by Rosenow,⁶⁷ that streptococci can invade the gallbladder wall in the form of emboli. The work of this writer leads, however, to a consideration of the last possibility by which bacteria can reach the biliary tract, namely, the lymphatics. From an experimental standpoint this route is of no importance. It is possible that the observations of Ledingham (see Morgan⁵⁰), who noted typhoid bacilli in the gallbladder of guinea-pigs that had been injected intraperitoneally, can be explained on this basis. Clinically, it is an established fact that in the course of peritonitis or other abdominal infections (appendicitis) streptococci can be transported through the lymphatics to the biliary passages. The lymph vessels of the pancreas, periduodenal and peripyloric tissues, end in the fossa transversa of the liver; infection of the extra hepatic biliary system from these regions is therefore quite possible (Mix⁷⁵). In a number of typhoid experiments we observed the frequent secondary infection of the gallbladder of rabbits with streptococci. In these cases the bile was sterile, while the wall gave on proper cultivation an abundant growth of indifferent streptococci. Coinciding with the observations of numerous surgeons (Baron⁷⁶), we also noted in chronic cholecystitis of animals the displacement of *B. typhosus* by *B. coli* or by streptococci. A lymphatic invasion is the most likely route of these infections. The fact that the lymphatic spaces and vessels of the rabbit's gallbladder

⁷⁵ Illinois Med. Jour., 1914, 25, p. 17.

⁷⁶ Beitr. z. klin. Chir., 1912, 77, p. 447.

or the extrahepatic ducts are seriously injured as a result of bacterial growth in the cystic bile or in consequence of mechanical injury to the biliary system lends support to this view. The so-called ascending route of infection is in all probability due to an invasion of bacteria through the lymphatics. The experiments of M. Müller,⁷⁷ with the typhoid bacillus in mice, moreover suggest that the spleen, liver, and their adnexa can readily become infected by way of the lymph stream. In animals with a native immunity against the micro-organisms this mode of invasion is the rule.

In summarizing these general considerations, it must be concluded that for the experimental production of a cholecystitis with intestinal organisms, such as the typhoid-dysentery bacilli and cholera vibrios, the hemato-hepatogenous route is the only one which has been definitely proved. An infection through embolic invasion of the wall may occasionally occur. This route demands, however, further experimental proof. An ascending infection follows, as a rule, the lymphatics; secondary or superimposed infections with streptococci take place through the same channels. The mechanism of bacterial transit from the interlobular veins or branches of the hepatic artery to the bile capillaries, and the factors which lead to a persistence or disappearance of the eliminated bacteria in the cystic bile and gallbladder, are as yet unknown. The facts presented later were collected with the desire to solve some of the questions which suggested themselves in the course of the critical analysis of our present knowledge concerning the problem of experimental typhoid cholecystitis in laboratory animals.

OPERATIVE PROCEDURES AND METHOD OF COLLECTING HEPATIC DUCT BILE FROM LABORATORY ANIMALS

For the experiments to be recorded in this and subsequent papers the sterility of the bile samples to be tested is of utmost importance; it is therefore self-explanatory that the operative procedures are conducted under strict asepsis. All animals used are completely etherized. To facilitate the exposure of the hepatic duct a thick pad is placed under the vertebral column between the costal and lumbar vertebrae. The shaved skin is thoroughly cleansed with soap and water, alcohol, ether, and painted with tincture of iodine. The body of the animal is always

⁷⁷ Ztschr. f. Fleisch. u. Milchhyg., 1911-12, 22, p. 106; Centralbl. f. Bakteriöl., I, 1912, 62, p. 335.

covered by sterile linen sheets and the field of operation is blocked by sterile towels, as customary for laparotomies.

As a rule, a medium incision not longer than 5 cm. is made, extending from the xiphoid cartilage to the umbilicus. Occasionally in rabbits the viscera are exposed by a transverse incision extending along the rib margin of the right side. The latter method has, however, no advantage over the median incision. After the peritoneum is opened the left hand of the operator grasps with three fingers the pyloric region of the stomach and by bringing this portion gently into the opening of the abdomen, the hepatic duct is made visible. Particular care is necessary that during this act of the operation no mesenteric blood vessels are torn. The common duct is made accessible by fixing the small intestines with gauze pads soaked in warm saline. The mesentery of the duct is cut, avoiding the blood vessels, and a threaded French needle passed under the duct about 0.5 cm. from the duodenum. The tied thread enables the assistant to put the duct on the stretch. A second silk thread is then placed about 1 to 1.5 cm. from the first ligature; the duct is transversely incised with a small pair of sharp scissors and usually without any difficulty a glass cannula with a good neck can be inserted. The thread is then tied on the neck of the tube. When the cannula is properly placed, perfectly clear, slightly yellowish-green bile enters it as soon as the first ligature which holds the duct on a stretch is released. The gall cannula is connected by a fairly stiff rubber tubing, which is brought out either through the abdominal incision or through a stab wound on the right side of the abdominal wall. A series of silk sutures close the peritoneal cavity. The wound is covered with collodium.

The animal is then fixed on a padded and electrically warmed board. The rubber tube is connected with a sterile glass tube in a two-holed stopper, the latter being inserted in a pyrex test tube or an alkaline-free graduated centrifuge tube. The glass and rubber connections hold about 0.8 to 1.0 c c of bile.

The operation is easily performed on rabbits, and provided properly made cannulas are used, not only contamination but also admixture of blood is regularly avoided. Of about 80 rabbits operated on, with the technic and the aseptic precautions mentioned, one or two bile samples only revealed contaminating staphylo- or streptococci. In guinea-pigs, the technic of exposing the hepatic duct without hemorrhage is considerably more difficult, and blood-thinned bile specimens occurred

in about 10% of the 30 animals operated on. As will be shown later, rats can be successfully prepared for hepatic bile collection, but the rate of flow is so slow that the animals operated on have to be kept in a fixed position for more than 12 hours. This prolonged period of collection produced in the two instances attempted, specimens contaminated by staphylococci.

The preparation of hepatic duct fistulas in dogs, cats, monkeys and goats offered no difficulties and in every instance sterile samples were obtained. It was, however, noted that the manipulations of the duodenum and of the liver caused regularly a prolonged period of reflex which inhibited bile secretion. With most of our dogs and cats we failed to obtain the necessary amount of liver bile without the use of cholagogues given either in form of ox bile by stomach tube 2 hours before the operation or more advantageously by injecting intravenously from 1 to 2 gm. of sodium taurocholate in warm saline solution. The cholagogue effect lasted usually from 2 to 3 hours; prolonged experimentation necessitated repeated injections. We are conscious of the fact that such procedures materially altered the constituents of the secretion, as will be shown later. The observations to be recorded on these biles should be viewed from this standpoint. The late Miss Foster of this laboratory has shown that about 90% of the injected taurocholic acid is eliminated in the fistular bile during the 2 to 4 hours following its injection. Numerous attempts to use bile obtained from dogs with simple gallbladder fistula were unsuccessful. The samples were regularly so badly contaminated with various micro-organisms that, in order to prepare suitable test specimens, repeated heating was necessary. The study of such samples naturally introduced new factors, which were not in the scope of our inquiry, and which dealt primarily with the bile freshly secreted from the liver.

In a number of rabbits the cystic duct was doubly ligated and cut before placing the cannula in the common duct. This operation is admittedly very delicate; utmost care is necessary to avoid injury or ligature of the cystic artery. The majority of our attempts resulted in hemorrhagic infarction, or minor circulatory disturbances in the veins of the gallbladder wall, which either became totally necrotic or showed hemorrhages and escape of blood into the lumen of the viscus. It is obvious that the injured tissues offer an excellent opportunity for localization of the intravenously inoculated typhoid bacilli. Our observations on the embolic invasion of the capillaries of the gallbladder

mucosa were made on rabbits, which were neither laparotomized nor exposed to injury by ligation of the cystic duct or common duct.

The selection of the rabbits for the experiments to be reported followed the principles outlined in previous papers. If possible, members of the same litter were chosen. In about 1% of the rabbits and guinea-pigs employed spontaneous cholecystitis either due to *B. coli* (2) or streptococci (3) staphylococci (1) impaired the value of the experiment. The cystic bile was in these instances either distinctly changed in color or sections of the thickened wall revealed a diffuse lymphocytic infiltration of the mucosa and submucosa. Occasionally the bile appeared to be normal, while the histologic picture exhibited a low grade infection of the lymphatics of the mucosa and subserosa. The preparation of the suspension of typhoid bacilli to be injected, the cultivation of the bile and blood specimens and the numerical determination of the bacteria in the tissues were treated in the same manner as stated in previous papers.

EXPERIMENTAL DATA

From the discussion of the present knowledge concerning the possible path by which the typhoid bacilli on intravenous inoculation reach the gallbladder, it is evident that the hemato-hepatogenous system is probably the usual route. The factors which favor the transit of the bacteria from the blood to the bile are, however, incompletely investigated. Moreover, nothing is known relative to the exact number of typhoid bacilli, which enter the biliary system in this manner, and their fate in the biliary secretion. The experiments thus far published (with the exception of those by Nichols) deal with the phase of the problem only in a qualitative manner. We are by no means convinced that the mere presence of bacteria in the bile, after an intravenous injection of typhoid bacilli in a sufficiently large number to ensure passage into the excretion, leads always to a cholecystitis or even to a temporary persistence of the organisms somewhat analogous to the human carrier state. Furthermore, one should know how soon after the injection and for what period of time the bacilli are discharged in the bile. In the preceding paper the fate of the typhoid bacilli was determined by cultivating the tissue from 1 to 160 hours after the injection. No attention was paid at that time to the behavior of the bacilli in the blood stream during the first hour. Bull ⁷⁸ and others have already considered

⁷⁸ Jour. Exper. Med., 1914, 20, p. 237; 1915, 22, pp. 475 and 487; 1916, 23, p. 419; 24, p. 25.

this phase of the problem, but in connection with the study of the disappearance of the leukocytes from the peripheral blood stream of animals with biliary fistulas that had been intravenously inoculated with living typhoid bacilli, the fate of these organisms in the blood vessels was simultaneously investigated.

The work of Nichols suggested a relationship between immunization and elimination of the injected bacteria. In the experimental study of this phase the results of this writer have in part been confirmed and amplified by our work on guinea-pigs.

THE ELIMINATION OF TYPHOID BACILLI IN THE HEPATIC DUCT BILE OF NORMAL AND IMMUNIZED RABBITS, GUINEA-PIGS AND DOGS

Charts 1 and 2 illustrate the rate of elimination of typhoid bacilli in the hepatic duct bile collected periodically from common duct fistulas of 2 normal and 2 immunized rabbits. The degree of leukopenia and the rate of disappearance of the inoculated bacilli are also demonstrated. The observations have been collected from a series of 8 successful experiments on coccidiosis-free animals. The cystic duct has always been tied before placing the hepatic duct cannula, and the animals have been permitted to recover from the operation (at least 2 to 3 hours) before the bacteria have been inoculated. Varying amounts of cultures grown on agar have been introduced intravenously. When the number of bacteria injected has been less than 1,000 million, the plates prepared from the bile specimen collected for a period of 2 hours have never shown more than 10 bacteria (table 1).

TABLE 1

ELIMINATION OF TYPHOID BACILLI IN HEPATIC DUCT BILE IN IMMUNIZED AND NORMAL RABBITS

Experiment	Rabbit	Number of Typhoid Bacilli Inoculated	Total Colonies in Bile	Time of Collection
1	1114a Immunized.....	600,000,000	6 in 21.9 c c	120 Min.
	1114b Normal.....	600,000,000	7 in 24.1 c c	120 Min.
2	1147a Immunized.....	10,000 million	12 in 20.35 c c	120 Min.
	1147b Normal.....	10,000 million	28 in 25.9 c c	120 Min.
3	1141a Immunized.....	12,000 million	13 in 15.3 c c	60 Min.
	1141b Normal.....	12,000 million	183 in 15.6 c c	60 Min.
4	1148a Immunized.....	20,000 million	23 in 7.4 c c	120 Min.
	1148b Normal.....	20,000 million	422 in 10.7 c c	120 Min.
5	1136a Immunized, 1½ year old...	24,000 million	3,377 in 36.6 c c	120 Min.
	1136b Normal, 1½ year old.....	24,000 million	24,609 in 27.0 c c	120 Min.

Inoculations of large doses varying from 10,000 to 24,000 million produce a rapid discharge of typhoid bacilli. In one instance it was profuse. With the exception of exper. 2, the general character of the

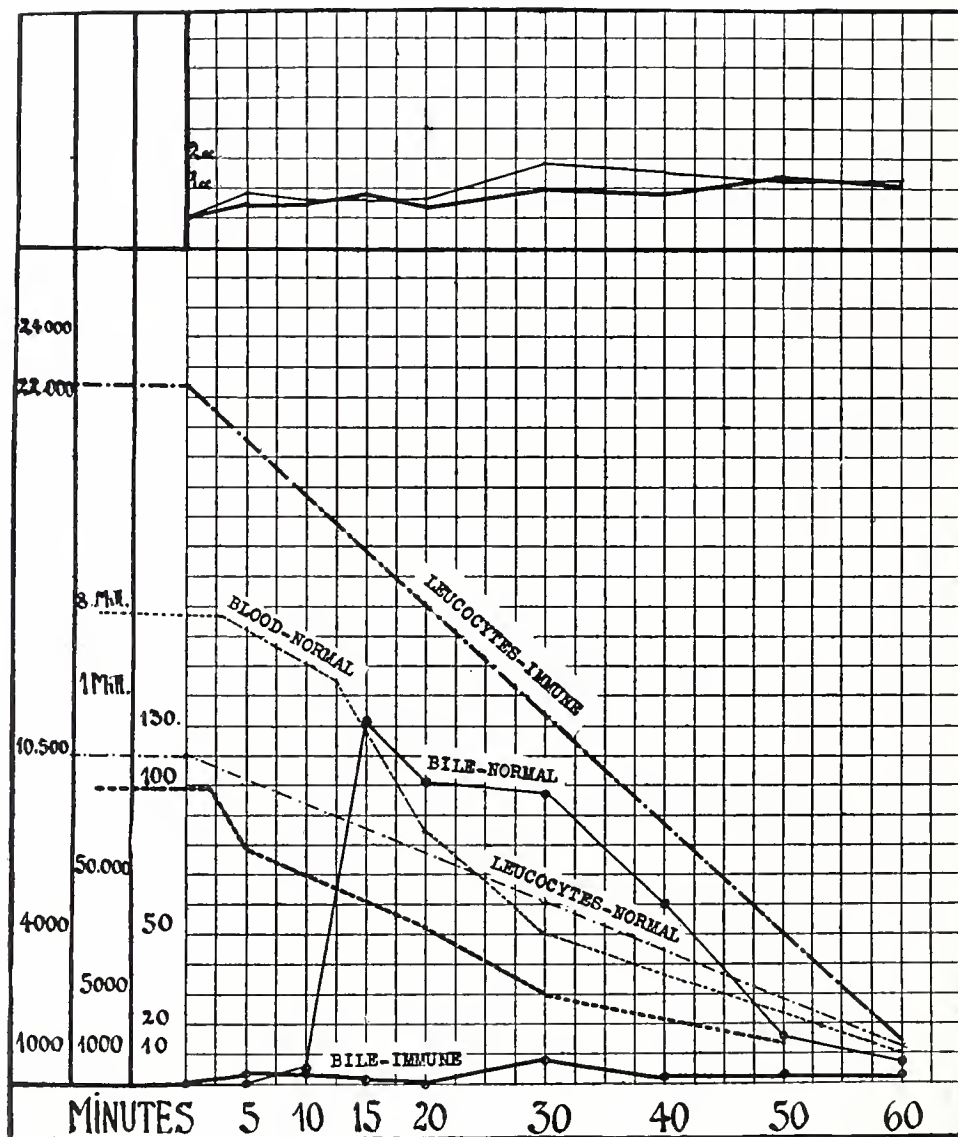


Chart 1.—Rate of elimination of typhoid bacilli in hepatic duct bile in 1 normal and 1 immunized rabbit. Degree of leukopenia and disappearance of bacilli from bloodstream. Rate of bile flow during the experiment.

curve of elimination has followed the one given in chart 1. In the normal animal the bacilli appear in greater numbers than in the immune. There are some differences in elimination characteristics for certain

litters, depending on the age of the individual animals. The age of the culture, whether grown with or without rabbit blood, has no influence on the total number of bacilli discharged in the bile. In every instance but one the cystic bile has been sterile.

As a rule, the elimination of the bacilli is immediate. Making an allowance for the bile which is present in the cannula at the time of the intravenous injection, it is obvious that less than 5 minutes elapse before the bacteria reach the biliary passages. Moreover, the maximum discharge occurs in the first 5 to 15 minutes, in some instances even in less time. In subsequent periods the number decreases rapidly and not infrequently ceases completely at the end of 1 hour. While these observations confirm the facts already reported by v. Fütterer, Biedl and Kraus, Blumenthal and Nichols, in very exceptional cases, as has been exemplified in the data published by Doerr, and by ourselves (paper IV), the elimination is delayed or even on repeated inoculation is not demonstrable. According to one of Doerr's tables (footnote 1, page 629), at least 8 hours elapsed before the typhoid bacilli inoculated in an amount of 2 standard loopfuls, an average of 5,000 million organisms, were present in the cystic bile of rabbits. Three of his animals in the same series killed on the 2nd, 4th, and 6th hours gave sterile cystic bile specimens. Doerr has failed to state whether the secretions have only been plated or whether they have also been enriched. On several occasions it has been noted that even repeated inoculations of large doses of typhoid bacilli have resulted either in sterile hepatic duct bile specimens, or the number of organisms demonstrated by plating has been below 10 and has been insignificant in proportion to the inoculum. This statement is best illustrated by the presentation of an experiment.

Exper. 9.—Normal rabbit 1059 (weight 2,525 gm.) was inoculated with 7,750,000,000 typhoid bacilli (polyhomogenous 5 strains) after his cystic duct had been ligated and a common duct fistula placed. During the initial period of 60 minutes no bacilli were discharged. At the end of the first hour an additional inoculation of 16,000,000,000 bacteria was made and the collection of the samples continued for 60 minutes. A total of 7 colonies was counted in specimens obtained 100, 110 and 120 minutes after the first injection. The collecting tube contained 2 organisms, while 0.8 c.c. of deep green, blood-free cystic bile gave 396 colonies. The gallbladder wall registered 23,000 typhoid colonies per 100 mg. of tissue.

The leukocytes dropped from 18,000 to 1,300 per c.c. and the blood freed itself of the bacteria as follows: 1 minute after the 1st injection, 1,400,000 per c.c.; 60 minutes after the injection, 30,000 per c.c.; 1 minute after the 2nd injection, 19,000,000 per c.c.; 60 minutes after the injection, 660,000 per c.c. in the peripheral blood and 100 c.c. in the heart blood.

It is evident from this and similar experiments that (1) even a large dose of typhoid bacilli in a limited number of rabbits never leads to a discharge of the bacilli by way of the hemato-hepatogenous route; (2) the gallbladder bile may become infected even after ligation of the cystic duct; and (3) particularly if one considers the tremendous dosage and the rapidity with which the blood stream freed itself of organisms, the transit of typhoid bacilli in the liver from the blood to

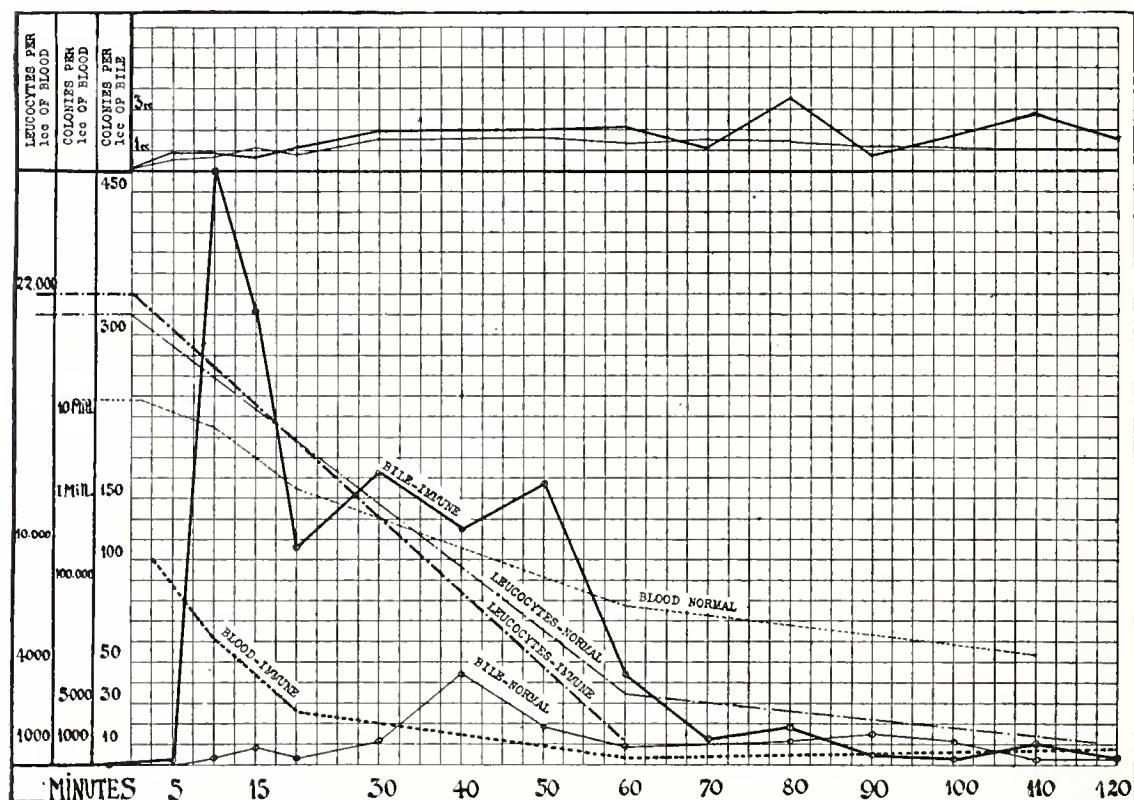


Chart 2.—Elimination of typhoid bacilli in the duct bile of a normal and of a recently immunized rabbit.

the bile capillaries in the first 2 hours after the injection cannot be the outcome of a mechanical rupture of the capillary walls by the masses of bacteria resulting in a flushing of the organisms into the biliary secretion.

Additional experiments confirm the foregoing conclusions as far as they concern the infection of the gallbladder whose cystic duct has previously been ligated. Inoculations of large doses of typhoid bacilli may cause an infection of the cystic bile in animals with a ligated cystic duct. Our experiments were originally conducted according to the procedure of Doerr. Nine rabbits were operated on; the cystic duct was carefully tied; and the animals permitted to

recover from the laparotomy. After a lapse of from 4 to 6 days, they were inoculated with a sublethal dose of *B. typhosus* (varying from 2 to 4,000 million). Twenty-four to 72 hours after the injection, the animals were killed and their tissues cultivated. Five animals must be eliminated from consideration on account of the profound changes in the gallbladder wall (hemorrhages, partial necrosis, etc.). Three of these rabbits had sterile cystic biles while the remaining 2 showed varying numbers of typhoid bacilli. Four rabbits were successfully operated on; the bile at necropsy was deep olive green and

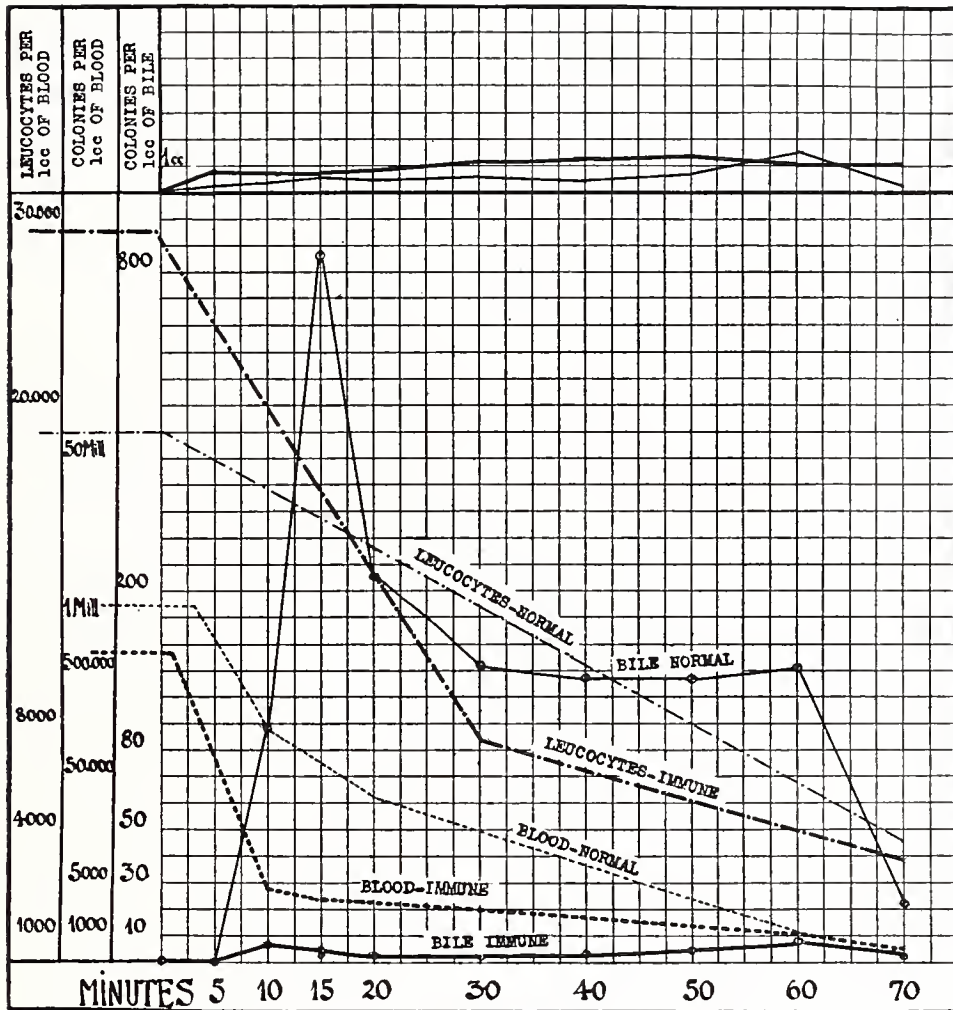


Chart 3.—Elimination of typhoid bacilli in the duct bile of a normal and of an immunized guinea-pig.

viscid; the gallbladder wall was microscopically intact. Neither of these animals gave a positive cystic bile culture and the gallbladder wall was either sterile or contained only a few bacilli. Moreover, the duodenal content and mucosa of these rabbits was sterile or failed to give specific colonies. On first consideration these experiments seem to confirm the experiences of Doerr

and Blumenthal, but on further comparison with other data at our disposal considerable hesitancy is felt in drawing far-reaching conclusions. The absence of typhoid bacilli in the duodenum deserves in this connection some consideration. Laparotomized rabbits succumb readily to a typhoid intoxication in the first 12 to 24 hours, as has been stated repeatedly. It is technically impossible to infect such animals with recently isolated typhoid strains in amounts which ensure elimination in the hepatic duct bile and which lead to an invasion of the gallbladder wall. In numerous experiments on rabbits which were not previously operated on it was noted that only 40% of the animals developed infected gallbladders, when injected with the same technic as in the above tests. The experimental data recorded in animals previously laparotomized are therefore seriously invalidated and cannot be used in any argument against the theory of embolic wall infection. Exper. 9, previously described, suggests a means of overcoming these technical difficulties. In these experiments a number of rabbits were laparotomized, the cystic duct carefully dissected, ligated and cut, and a common duct cannula inserted. After a lapse of from 3 to 5 hours, they were injected intravenously with large amounts of typhoid bacilli (15 to 24 billion, one slant of peptic digest of $\frac{1}{2}$ slant of rabbit-blood agar cultures). Bile specimens were collected for from 2 to 8 hours, and at the end of this period the animals were killed. Careful cultures revealed in 2 of the 6 rabbits successfully operated on, 69 and 117 typhoid colonies in 0.4 and 0.7 c.c., respectively, of deep green bile. Neither of the 2 specimens contained traces of blood; the gallbladder walls gave 8,700 and 6,900 colonies, respectively, per 100 mg. of tissue. During the observation period of 8 hours, 3,300 and 1,280 typhoid bacilli were eliminated by the liver. These experiments suggest that in a number of rabbits typhoid bacilli can reach the cystic bile by way of the blood vessels of the gallbladder wall provided a sufficiently large inoculation is given. The manner in which the bacilli enter the bile will be discussed in connection with the consideration of the microscopic findings in these gallbladders.

It is definitely shown in chart 1 and table 1 that the immunized rabbit discharges less organisms than the normal animal. This fact is even more strikingly shown in chart 3 illustrating the same type of experiment in a normal and immunized guinea-pig. The graph has been prepared from the data of an experiment selected from a series of 4. The study conclusively proves that in the immunized guinea-pig either no bacteria or few pass into the biliary capillaries. The result depends entirely on the number of bacilli inoculated, as is shown by the following figures:

TABLE 2
NUMBER OF BACTERIA IN HEPATIC DUCT BILE OF IMMUNIZED AND NORMAL GUINEA-PIGS
AFTER INOCULATION OF TYPHOID BACILLI

B. Typhosus Injected	Time after Injection	Immunized	Normal
100,000,000	120 Minutes	0	2,216
300,000,000	60 Minutes	11	1,942
450,000,000	60 Minutes	33	239
600,000,000	60 Minutes	45	1,680

As the immunized guinea-pigs can destroy typhoid bacilli more readily than the normal animal, a difference which is not demonstrable in the rabbit, it is not at all surprising to find the contrast we have noted in the elimination of bacteria. Immunization obviously produces in the blood vessels of the liver some factors controlling the elimination of the organisms which can only be overcome by a large inoculation. The nature of these factors is not definitely established, but we have been able to demonstrate in the guinea-pig histologically a very active phagocytic action of the endothelial cells. At the end of 2 hours most of the Kupffer cells are packed with typhoid bacilli. The same phenomenon occurs also in the rabbit but to a less marked degree. The endothelial lining of the liver capillaries, which is endowed with the maximum ability to phagocytize and to destroy typhoid bacilli, will also be resistant to the typhoid toxins, which tend to injure the capillary wall and permit in this manner an escape of some bacteria into the adjacent biliary capillaries. The guinea-pig is relatively insusceptible to the typhoid toxin⁷⁹ present in the bacterial emulsion. The maximum elimination of the bacteria in the hepatic duct bile occurs in the first 10 to 25 minutes after the injection, corresponding to the time when the liver has accumulated approximately from 25 to 40% of the bacterial masses inoculated. In this connection one recalls the observations of Helly⁸⁰ and of Schwarz,⁸¹ who have found typhoid bacilli not only inside, but outside of the capillaries in the pulp cords and the malpighian bodies of the spleens of guinea-pigs 10 minutes after an intravenous injection. The capillary system in general, and not only that of the liver vessels, is therefore permeable to bacteria shortly after a drastic intravenous injection. In the immunized guinea-pigs polymorphonuclear and endothelial phagocytosis begins immediately, and comparatively few organisms can be found free in the blood stream. In accordance with this fact, the number of typhoid bacilli per c c of peripheral blood of the immunized guinea-pig is less in the first 20 minutes than in the normal animal. As long as the bacterial masses to be phagocytized are comparatively small and can be handled by the cells destined for this purpose, no bacteria will enter the bile. In case the injection and the subsequent hepatic invasion are overwhelming, a transit of organisms will be recorded. The elimination is apparently

⁷⁹ For a detailed consideration of the so-called typhoid toxin consult the recent summary of H. Zinsser, *Jour. Immunol.*, 1902, 5, p. 265.

⁸⁰ *Beitr. z. path. Anat. u. z. allg. Path.*, 1903, 34, p. 387.

⁸¹ *Ztschr. f. Heilk.*, 1905, 26, p. 295.

not an excretory process. This statement is based on the following observation. The foregoing experiments were repeated after adding to the bacterial suspension a 1% Congo-red solution in saline (1 c.c. in guinea-pigs and 10 c.c. in rabbits, Cecil and Weil⁸²). The dye appeared in the normal and immunized animal in from 4 to 18 minutes. No differences in the amounts of the stain excreted could be determined in the 2 animals, but numerous typhoid bacilli were present in the bile collected from the normal, while sterile plates were obtained from the immunized guinea-pigs. In some instances the excretion of the dye preceded the appearance of bacteria in the bile by from 8 to 15 minutes. The results just mentioned suggest that a further study of the transit of bacteria from the blood to the bile capillaries be combined with an analysis of the excretory function of the liver of the same animal by some similar test: All these observations constitute a strong argument in support of the conception of a cellular immunity, particularly when tests are conducted on guinea-pigs which have been vaccinated several months previous to the tests and which have lost their serum immune bodies. The number of such experiments is relatively small, but definitely indicates that a guinea-pig once treated with typhoid bacilli will always behave like an immunized animal even when the usual tests suggest that his immunity has been lost. A persistent experimental typhoid cholecystitis can only be produced in immunized guinea-pigs with very heavy intravenous infections.

In the rabbit the endothelial cells of the liver capillaries function less vigorously than in the guinea-pig, but differences in activity of the cells of the normal and of the immunized animals can be detected by the bile elimination test, provided a sufficient time has elapsed between the last immunizing dose and the test injection. Immunized rabbits injected with small doses of typhoid bacilli on the 6th (chart 2) or 10th day (Nichols, footnote 27, 504) discharge more bacteria than normal ones. The endothelial barrier is apparently imperfect; the cells have in consequence of the vaccination not sufficiently regenerated or acquired the function of "immunity," and bacteria leak in to the bile capillaries. The observation has been confirmed repeatedly. Irrespective of the agglutinin content of the blood or the number of injections, the bile of immunized rabbits infected before the 6th to 10th day contains more bacilli than the same secretion of normal rabbits.

⁸² Jour. Am. Med. Assn., 1917, 69, p. 521.

For example, rabbit 1374 was immunized intravenously by 10 inoculations of heat-killed typhoid bacilli. Six days after the last injection, when the agglutination titer was 1:200,000, the animal, together with rabbit 1090 (a "recovered carrier" with an agglutination titer of 1:200) was inoculated with 8.5 billion living typhoid bacilli. Killed after a lapse of 2 hours, the bile of rabbit 1374 contained 800,000 typhoid bacilli per 3.3 c c, while 1.2 c c bile of rabbit 1090 proved sterile on direct plating and 0.4 c c of the same on enrichment in broth was likewise sterile. The destruction of the bacteria in the tissues of both animals was practically identical. The blood contained 200 and 300 typhoid bacilli per 1 c c of blood.

Our experiments fully explain one observation made by Nichols. One of us (K. F. M.) immunized the rabbit used by this writer. According to the available records, the test injection was given on the 5th day after the last immunizing inoculation. The conclusion of Nichols, that more bacilli appear in the bile after an injection of the same dose in immunized animals than in normal animals, deserves some modification. It should read "immunized animals which have not recovered from the treatment discharge more bacilli." The facts elucidated by the experiments may have some bearing on the observations of Metchnikoff and Besredka,⁸³ that anthropoid apes are not protected against an alimentary typhoid infection when the animals are tested in from 4 to 6 days after finishing the immunizing treatment. They partially explain our own experiments reported in paper III: namely, immunized rabbits infected on the 10th day after the injection of the last dose of vaccine develop in a higher percentage of instances gallbladder infections than nonprotected animals. It is not unlikely that the reports of Creig⁸⁴ and Flu⁶² on rabbits developing gallbladder infections in the course of immunization with cholera-like vibrios and Flexner dysentery bacilli may in part be explained by the foregoing findings. The question naturally arises, what constitutes a safe period for recovery? Until recently, all processes of immunity have been gaged by the appearance or disappearance of immune bodies in the blood serum, while little or no attention has been paid to the reparative changes which by necessity must take place in the injured tissues. Nobody will deny that heat-killed typhoid vaccines produce, for example, liver necrosis, proliferation of macrophages, etc., but how many days are necessary to cause a *restitutio ad integrum* fails to interest the serologist, who studies disease and infection only by test-tube experiments. It is a matter of common knowledge in our laboratory that

⁸³ Ann. d. Inst. Pasteur, 1911, 25, p. 193.

⁸⁴ Indian Jour., Med. Res., 1915-16, 3, pp. 259 and 397.

a rapid method of immunization, for example, the procedure suggested by Fornet and Müller, by Bull⁸⁵ and by others, produces immune serums of great potency with no loss in animals and no gallbladder infections. On the other hand, the "standard method" of immunization, at 7 to 10 days, in comparison furnished poor serums, frequent losses of animals from cachexia and numerous gallbladder infections. The rapid method of immunization of rabbits finds, therefore, some advantageous justification in view of these data.

The elimination of typhoid bacilli in the hepatic duct bile of dogs has been studied in 10 animals. The results have been inconstant. Differences between the rate and degree of bacterial elimination in immunized and normal dogs have been indefinite. Injections of less than 8,000 million have failed to cause transit of bacteria, in dogs varying in weight from 14 to 26 pounds. Cats behave in a similar manner. It is generally known from the studies of Cushing⁸⁶ and our own, that typhoid bacilli introduced directly into the cystic bile of dogs disappear rapidly and that a cholecystitis can only be produced by considerable injury of the wall or by placing a foreign body in the gallbladder (Marxer¹⁶). A more careful study of the mechanism of gallbladder infection in these species of animals has therefore been considered superfluous.

THE DISAPPEARANCE OF THE TYPHOID BACILLI AND OF THE LEUKOCYTES FROM THE PERIPHERAL BLOOD STREAM

Blood samples were collected from the ear veins at regular intervals during the experiments on fistula animals. By means of pipettes with a capacity of 0.01 c.c. the blood drops collecting on a sterilized area of the ear were aspirated. Leukocyte counts were obtained from the same specimens. For each test a fresh incision was made. Hypocoagulability favored the collection of blood and as long as the blood pressure was normal, uncontaminated cultures were obtained. The findings are included in charts 1, 2 and 3.

In a general way, the observations confirm the well-known fact, originally established by Wyssokowitch⁷² and von Fodor,⁸⁷ that bacteria intravenously inoculated disappear rapidly from the circulation. The analysis of the data presented in paper IV indicates that at least one

⁸⁵ Jour. Exper. Med., 1916, 24, p. 25.

⁸⁶ Bull. Johns Hopkins Hosp., 1899, 10, p. 166.

⁸⁷ Arch. f. Hyg., 1886, 4, p. 129; Von Fodor, I., and Rigler, E.: Centralbl. f. Bakteriol., I. 1898, 23, p. 930.

hour is necessary to cause a complete absence of the typhoid bacilli from the heart blood, obtained from living animals by puncture. Furthermore, it has been shown that the organisms are deposited in the liver, spleen, bone-marrow and lung. At the end of from 1 to 2 hours the bacteria may reappear and circulate in moderate numbers until the 4th to 8th day. In paratyphoid infected animals the period is even longer and in case the animals succumb the bacilli may be demonstrated in the blood in large numbers at the time of death. The disappearance of the typhoid bacilli is decidedly more rapid in the immunized animals, but not as complete as demonstrated by Bull. Our results differ from those reported by this writer for these reasons: (a) a heavier dose of bacteria was injected; and (b) the blood samples were collected from the peripheral vessels instead of from the heart.

The mechanism of the removal of bacteria which is the same even on repeated injections (exper. 9) has been the subject of considerable study. It is interpreted by Bull⁸⁸ to be a result of *in vivo* agglutination, but recently Debres and Govaerts⁸⁹ attribute the clumping of the intravascular bacteria to the action of the blood platelets. The intravenous inoculation in their opinion causes a disturbance of the plasma with an agglomeration of platelets, which in turn engulf the bacteria. The agglomerated masses vanish from the blood stream to be deposited subsequently in the capillaries, sinusoids, etc., of the organs. The removal of the blood platelets produces in turn a hypocoagulability. We have had occasion repeatedly to note the prolongation of the coagulation time of the blood collected 15 to 20 minutes after the inoculation of living or dead typhoid bacilli. The following facts also speak against an *in vivo* agglutination. According to Ten Broeck⁹⁰ and our own observations, virulent and invasive hog cholera or paratyphoid bacilli are rapidly clumped and disappear from the blood even though the blood contains no demonstrable agglutinins, while Hopkins and Parker⁹¹ noted rapid removal of virulent hemolytic streptococci without any evidence of agglutination. *In vivo* agglutination may be positive even when *in vitro* experiments fail to demonstrate this property. It is the recognition of these exceptions which casts doubt on the correctness of Bull's interpretation of the mechanism explaining the phenomenon of removal of bacteria from the blood stream.

⁸⁸ Jour. Exper. Med., 1915, 22, pp. 475 and 487.

⁸⁹ Comp. rend. Soc. de biol., 1918, 81, p. 53; Presse méd., 1918, 26, p. 597.

⁹⁰ Jour. Exper. Med., 1917, 26, p. 441.

⁹¹ Ibid., 1918, 27, p. 1.

In normal guinea-pigs the bacilli are loosely aggregated without being truly agglutinated and yet they promptly collect in the lung, liver, spleen, etc. In a general manner one gains the impression from properly stained smears prepared from the organs that a true agglutination has not taken place, but that physical disturbances in the colloidal suspensions, the blood and the bacteria, are responsible for the aggregations of bacilli. In some respects it is difficult to distinguish agglutinations in vivo from the phenomenon of the concentration of foreign particles in the viscera observed by Werigo,⁹² in normal animals. In immunized rabbits and guinea-pigs the removal of bacteria takes place more rapidly, but we fail to note any differences in the formation of the clumps, which would definitely indicate true agglutination. As a rule, the aggregations are not more compact, nor do they consist of more bacteria. Our observations record the fact that the bacteria disappear from the circulation and that this disappearance is frequently followed in the normal animal by the discharge of the organisms into the hepatic duct bile. Whether this removal is the result of in vivo agglutination or the action of the blood platelets, or purely a dispersion phenomenon of the two colloids, has to be decided by further investigation.

The behavior of the leukocytes as illustrated in charts 1, 2 and 3, deserves some consideration, as it may assist in the explanation of the ultimate fate of the bacilli accumulated in the viscera. The observations made on animals which, on account of the hepatic duct fistula, exhibited a postoperative leukocytosis, confirm the well-known fact that the intravenous injection of bacteria or foreign protein causes a leukopenia in the peripheral circulation notwithstanding the postoperative leukocytosis. This condition is again followed in a few hours by a considerable leukocytosis. In the smears 10 to 15 minutes after the injection the majority of the white cells were mononuclear forms. The studies of Goldscheider and Jacobs,⁹³ Werigo,⁹² Schwarz,⁸¹ C. W. Wells,⁹⁴ Sellards and Baetjer,⁹⁵ Jolly,⁹⁶ Nagao⁹⁷ and others lead to the conclusion that the leukopenia is the result of the accumulation of the polymorphonuclear leukocytes in the internal circulation, especially in the liver, spleen and lungs. The view of an uneven dis-

⁹² Ann. de l'Inst. Pasteur, 1892, 6, p. 478.

⁹³ Ztschr. f. klin. Med., 1894, 25, p. 373.

⁹⁴ Jour. Infect. Dis., 1917, 20, p. 219; 1918, 20, p. 502.

⁹⁵ Bull. Johns Hopkins Hosp., 1918, 29, p. 135.

⁹⁶ Compt. rend. Soc. de biol., 1918, 81, p. 756.

⁹⁷ Jour. Infect. Dis., 1920, 27, p. 327.

tribution of the leukocytes in experimental typhoid infections is, however, not shared by Studer,⁹⁸ by Tachigara and Miura,⁹⁹ by Pepper and Miller,¹⁰⁰ and by others, mainly on account of their inability to demonstrate at the low point of the leukopenia an accumulation of leukocytes in the internal organs. The leukopenia is, according to Studer, who injected his animals subcutaneously, the result of a functional disturbance provoked by the typhoid toxin on the bone-marrow and on the lymphatic tissues. A similar opinion has been expressed by Tachigara and Miura. In this connection we recall some observations made in the course of studies on hyperleukocytosis (Hurwitz and Meyer,¹⁰¹) following the injection of living typhoid bacilli. During the period of leukocytosis the increase in the percentage of young forms of leukocytes or metamyelocytes is so great as to suggest that during the leukopenia some of the leukocytes are destroyed or that the typhoid bacillus injures the leukopoietic tissue, namely, the bone-marrow. This peculiar blood picture is frequently more marked in animals which have been injected with living typhoid bacilli. There are several explanations of the purpose of the localization of leukocytes in the viscera. The theory of a negative or repellant chemotaxis according to which the introduction into the general circulation of a foreign protein repels the polymorphonuclear leukocytes, causing them to find refuge within the capillaries of the internal organs, is theoretically possible. There is some doubt in the opinion of numerous observers as to the actual occurrence of a negative chemotactic action on leukocytes. The evidence adduced from our findings supports the conception of a positive chemotaxis in the viscera as advanced by C. W. Wells, Nagao (footnote 97, p. 351), and others. The disappearance of the polymorphonuclear leukocytes from the blood stream was frequently preceded by the clumping of the bacteria and their removal to the liver, spleen, etc. This fact is best shown in table 3.

Smears from the lung and liver demonstrate the well-known phagocytosis described by Bull.⁸⁸ It is obvious that the leukocytes perform a definite function, at least during the first 2 hours after an intravenous infection, in removing and distributing to the detoxicating organ—the liver—a considerable proportion of the typhoid bacilli. The bacterial protein liberated during the course of phagocytosis and

⁹⁸ Thesis, University of Zürich, 1903.

⁹⁹ Mitt. a. d. med. Fakult. a. k. Univers. z. Tokyo, 1917, 17, p. 539.

¹⁰⁰ Jour. Infect. Dis., 1916, 19, p. 694.

¹⁰¹ Jour. Exper. Med., 1916, 24, p. 515.

phagolysis may exert an additional chemotactic effect on the leukocytes or may in turn injure the bone-marrow, as suggested by Studer and others. The latter point can, however, not be decided from the data at our disposal, and the action does not occur during the first 2 hours.

This interpretation of the leukocyte counts affords no support for the conclusion that the leukopenia in typhoid fever is explained on the same basis. As far as the leukocyte counts of the peripheral blood is concerned, the available evidence indicates that the absolute diminution is much less than one has ordinarily supposed. Moreover, the spleen tumor is not the result of an accumulation of leukocytes as assumed by C. W. Wells, but due to a hyperplasia and activity of the reticulo-endothelial macrophages accompanied by a toxic inhibition of the leukopoietic function as stated by F. A. Evans,¹⁰² S. Gräff¹⁰³ and others. Moreover, the recent observations of Askanazy¹⁰⁴ and of Gräff indicate that the typhoid bacillus and its toxin exert primarily specific positive chemotactic influence on the endothelial cells, while the leukocytes only become engaged later in the course of the disease when the necrotic tissues saturated with bacterial protein act as chemotactic foreign bodies. This local accumulation of leukocytes for the purpose of digestion of the necrobiotic foci has no appreciable influence on the blood picture of the peripheral blood.

TABLE 3

RABBIT 1265 INJECTED WITH 345,000,000 TYPHOID BACILLI. 1 C.C. OF THE SERUM OF THIS RABBIT DESTROYS IN 5 HOURS 1,000,000 TYPHOID BACILLI

Time After Injection	Heart Blood Contained	
	Bacteria per C c	White Cells
30 seconds.....	3,200,000	11,300
2 minutes.....	800,000	14,600
4 minutes.....	400,000	10,500
10 minutes.....	34,000	
11 minutes.....	34,800	4,200
15 minutes.....	6,500	2,600
20 minutes.....	800	1,400

Experiments on rabbits even when conducted with paratyphoid bacilli cannot be chosen for a reproduction of the condition in man. The removal of the bacteria and the subsequent leukopenia resulting from an intravenous injection is nonspecific and can be observed following the injection of any micro-organism. Even the progressively

¹⁰² Bull. Johns Hopkins Hosp., 1916, 27, p. 356.

¹⁰³ Deutsch. Arch. f. klin. Med., 1918, 125, p. 352; and 126, p. 1.

¹⁰⁴ Askanazy, M.: Deutsch. med. Wehnschr., 1916, 42, p. 897.

invasive paratyphoid bacilli are actively phagocytized during the period of leukopenia, when the leukocytes and the clumped bacteria are brought in intimate contact. The ultimate fate of the gram-negative bacilli in the viscera may depend on some mechanism other than the ingestion by polymorphonuclears, namely, the endothelial cells or the bactericidal substances liberated by the leukocytes may play an important rôle in this problem.

CHOLECYSTITIS AS A RESULT OF GALLBLADDER WALL INFECTION

In the course of nearly 500 necropsies on rabbits infected by intravenous injection of typhoid bacilli, we noted in about 25% of the animals extensive inflammatory processes in the gallbladder wall. This organ was frequently adherent to the abdominal wall, the liver lobes, the stomach or even to the large intestines. Some rabbits killed from 8 to 10 days after the injection exhibited either a diphtheritic, necrotized or perforating gallbladder wall, the lesions being prominent particularly in the fundus region. Numerous small mural abscesses associated with hemorrhages were occasionally evident. In some experimental series the lesions named in the foregoing were more frequent than in others; in fact, in one series of 48 rabbits, the inoculation of agglutinated living typhoid bacilli produced in the gallbladder of 5% of the animals, blood-tinged biles with small or large blood clots. In a number of instances hemorrhagic ulcers or erosions were noted in the jejunum of the same animals. Histologically, the lesions originated from the terminal blood vessels of the mucosa. These and similar observations, the experimental results reported in paper IV, and the findings collected from rabbits infected intravenously with paratyphoid B. bacilli (Litch and Meyer⁴³) left no doubt that the gallbladder wall is often seriously damaged in the course of a typhoid or paratyphoid infection. Careful culture studies supplied, moreover, data which indicated that gallbladder biles might be found sterile, while the walls yielded innumerable specific colonies. Furthermore, the findings of a large percentage of cholecystitis cases among immunized rabbits did not harmonize with our conception of a hemato-hepatogenous route of infection. It was obvious that the small number of typhoid bacilli which was eliminated in the bile could pass directly through the hepatic duct into the intestines and might never reach the gallbladder. These and other considerations

made it apparent that the conception of a hemato-hepatogenous route of biliary infection did not explain all of our results. An attempt was therefore made to verify the studies of Chiarolanza,⁴⁸ who demonstrated nests of bacilli lying in relation to the capillaries of the gallbladder wall, from which source the bile was apparently invaded.

The experiments of J. Koch and Chiarolanza⁴⁸ have already been discussed and it has been concluded that for technical reasons they are open to serious criticisms, as are those dealing with the ligated cystic duct reported by R. Doerr.¹ A histologic study of the gallbladders removed at various periods after the infection has therefore been chosen as the most promising method of determining the possibility of infection of the bile by way of the gallbladder wall. Such an analysis gives not only definite information relative to the theory of wall infection, but also furnishes facts which help in the explanation of the exclusive persistence of typhoid bacilli in the gallbladder.

The literature contains reports of the histologic studies of Weinfurter,⁵⁸ Hailer and Ungermann,¹⁰⁵ Emmerich and Wagner,¹⁰⁶ Violle¹⁵ for typhoid, and those of Creig⁷³ for cholera cholecystitis of the rabbit. As a rule, the chronic stage of the infection has been studied. The majority of writers mention cellular infiltration with collections of bacteria in the mucosa, and accept unreservedly the descriptions and interpretations of Chiarolanza. An excellent description of the gallbladder lesions found in rabbits infected with a specific cholecystotropic paratyphoid bacillus has been published by Fränkel and Much.⁶⁹ Bacterial thrombi have been observed by these investigators in the capillaries of the mucous membrane either at the top or at the base of the folds. Frequently wall abscesses and bacterial masses have been noted in the lymph vessels. These reports are in marked contrast to the repeated statements of Nichols¹⁰⁷ that he has been unable to find bacterial foci in the gallbladder wall.

In the course of our infection experiments we systematically collected and fixed the gallbladders, while warm, in Zenker's solution. Sections were stained with hematoxylin-eosine, or with dilute Manson's blue or carbolthionine. The gallbladders of 132 rabbits have been examined, but a few characteristic sections are chosen for a discussion of the most important findings.

¹⁰⁵ Arb. a. d. k. Gsndhtsamte, 1914, 47, pp. 303, 451 and 470.

¹⁰⁶ Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

¹⁰⁷ Jour. Am. Med. Assn., 1917, 68, p. 958; Nichols, H. J.; Simmons, I. S., and Stimmel, C. O.: Ibid., 1919, 73, p. 680.

The description of the microscopic structure of the normal gallbladder, as reported by Violle¹⁵ and others, deserves one addition. In the majority of the gallbladders we found the villi of the mucosa located in the fundus portion to be long and irregular (fig. 1), resembling a papillomatous growth. The mucosa of the neck and the lateral walls, in contrast, have short and stumpy villi (fig. 2). The epithelium always exhibits a cuticular border, the cells are frequently of the goblet type, and in properly fixed material the lining is perfectly intact.

The pathologic changes, which we found in looking over many hundreds of sections, are definite and conclusive. In this connection it should be emphasized that the lesions to be described can only be found in 25% of the rabbits inoculated into an ear vein or by way of a radical of the portal vein. This statement applies particularly to the important lesions which develop between the 6th and the 72nd hour. The course of the wall infection can be discussed after studying the photomicrographs (in figs. 1 to 12), and is briefly as follows:

At the end of the 6th hour, after an intravenous injection of at least 5,000 million organisms in a rabbit weighing 2500 gm., the gallbladder bile may be sterile, but the wall may contain thousands of typhoid bacilli. A scrutiny of the villi of such a gallbladder may reveal dilated capillaries, perhaps nests of leukocytes and an edematous infiltration of the perivascular tissue (fig. 2). Typhoid bacilli can occasionally be demonstrated in the vascular endothelium, but quite regularly in the Kupffer cells of the liver. At the end of 24 hours a hemorrhage may occupy the tip of the villus and the adjacent epithelium is in a stage of necrobiosis. The cellular infiltration of the mucosa is confined to the necrotic villus (fig. 4). The bile contains culturally few bacteria, tinctorially a few clusters may be demonstrated. The focal necroses of the villi resemble the well-known lesions usually found in the liver of the same animals. The mucosa of the fundus is the place of predilection and the tissue destruction may be limited to one or two folds. It is self-evident that serial sections alone will reveal this type of wall infection. Toward the end of the 48th and 60th hour the focal necroses in the villi are in some animals quite numerous and may in certain cases be recognized macroscopically as small yellowish spots. The epithelium of the villi is frequently intact and shows normal nuclei. The photomicrographs (figs. 5 and 6) illustrate the location of the areas either in the top or the base of the villi. The center of the necroses consists of cellular debris, fibrin and

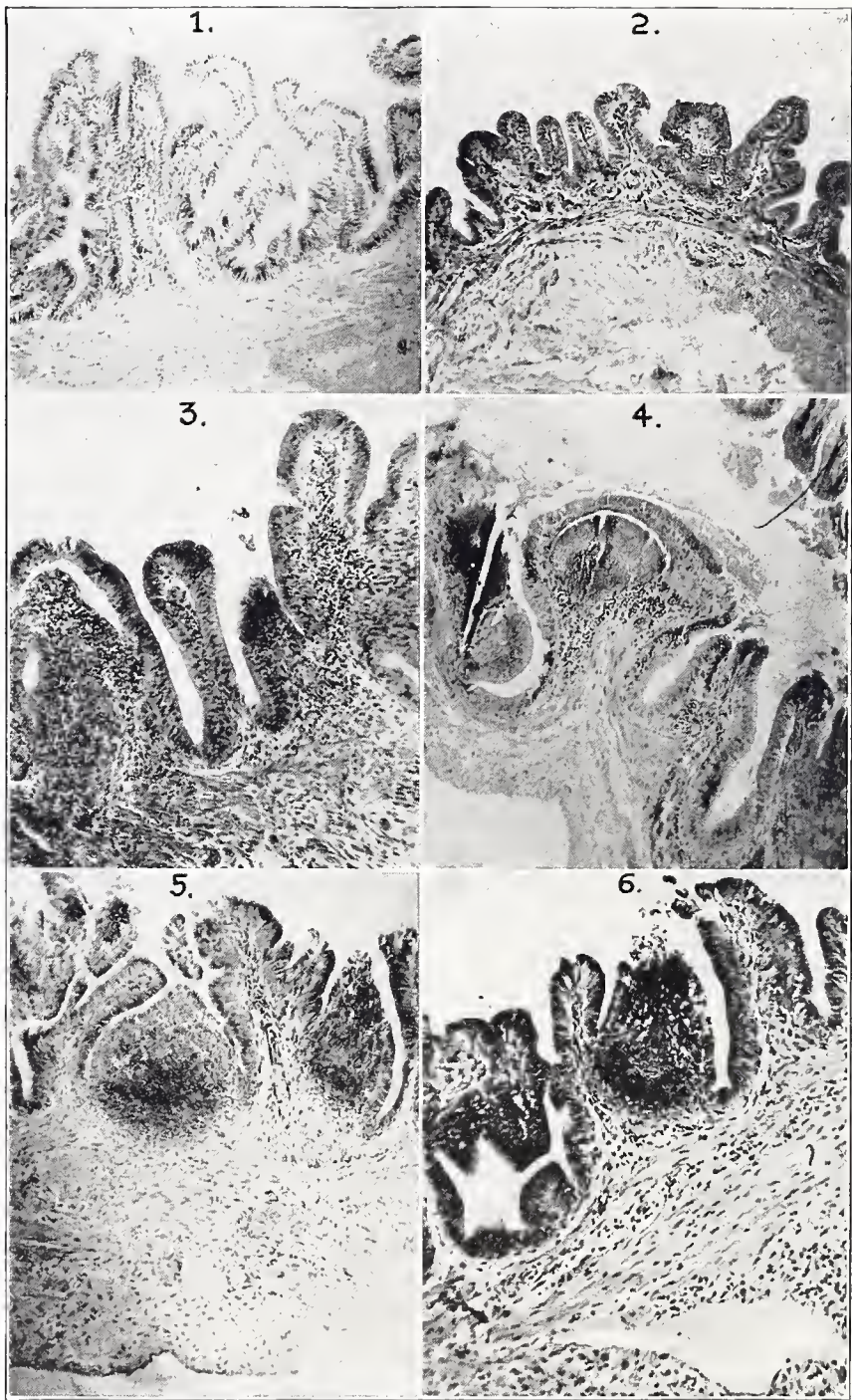


Fig. 1.—Rabbit 725; fundus of normal gallbladder.

Fig. 2.—Six hours. Rabbit 1701 A; intravenous 8 billion; green bile, sterile; wall innumerable colonies.

Fig. 3.—Twenty-four hours. Rabbit 1704. Direct gallbladder injection; 22 billion typhoid bacilli; colorless, limpid fluid with leukocytes, no blood.

Fig. 4.—Twenty-four hours. Rabbit 1707 (11). Six billion intravenous; bile deep green, viscid, 150 colonies per c c; wall ∞ colonies; macroscopically intact mucous membrane.

Fig. 5.—Rabbit 897; *B. typhosus* $\frac{1}{2}$ blood-agar slant; bile deep green viscid, ∞ colonies from bile and gallbladder; subserous edema; mucosa slight erosions.

Fig. 6.—Rabbit 897; *B. typhosus* $\frac{1}{2}$ blood-agar slant; bile deep green viscid, ∞ colonies from bile and gallbladder; subserous edema; mucosa slight erosions.

clusters of bacteria. Occasionally the demarcation and the discharge of the necrotic villi into the lumen of the gallbladder can be followed. Culturally at this stage of the infection the cystic bile may show one million bacteria per c.c. The presence of a large number of bacilli in the bile leads to an edema and a diffuse cellular reaction of the entire mucosa of the gallbladder, while the localized necrosis may develop into a diphtheritic tissue defect, which extends into the muscularis, submucosa and serosa. In some instances a fibrinous pericholecystitis, even a perforation of the ulcerative, gangrenous inflammation has been observed. The formation of hemorrhages, the focal necroses and the diphtheritic lesions on the tips and at the base of the villi definitely suggest an embolic invasion of the terminal capillaries.

Theoretically it is remotely possible that the typhoid bacilli, which reach the bile by way of the hemato-hepatogenous route, multiply in the stagnant content of the gallbladder and invade the villi from within. Moreover, it is not unlikely that the bacteria can destroy the epithelial lining and enter the mucous coat in consequence of these defects. As the entire conception of the transverse infection rests on the conclusive demonstration of a primary focus in the capillaries of the wall, we have inoculated large doses (1 to 4 billion) of typhoid bacilli in 0.5 c.c. of saline directly into the gallbladder of normal rabbits in order to determine whether the gallbladder foci described in the foregoing could be developed in a similar manner by this route. The injections have been made with and without ligature of the needle puncture according to the method of Venema.²³ Six animals successfully operated on without hemorrhage into the gallbladder, have been killed 24 to 60 hours after the intracystic injection. Serial sections showed a complete absence of localized areas of necrosis in the villi. The histologic picture (fig. 3) is characteristic for a catarrhal cholecystitis. The epithelium is intact, invariably studded with emigrating leukocytes, and sometimes it is loosened from the mucosa. The latter is diffusely infiltrated with lymphocytes and polymorphonuclear leukocytes. The inflammatory process involves the lymphatic spaces and lymph vessels of the submucosa and serosa; in fact, the entire gallbladder wall is macroscopically edematous. The dilated lymph vessels and capillaries contain thrombi of leukocytes and lymph material. The lymphostasis resulting from the involvement of the lymphatic system extends along the bile duct into the portal tissues of the liver. Neither necrosis of the tips of the villi nor diphtheritic ulcerations analogous to illustrations 4, 5 and 6 can be detected. The typhoid bacilli and their products invade the wall

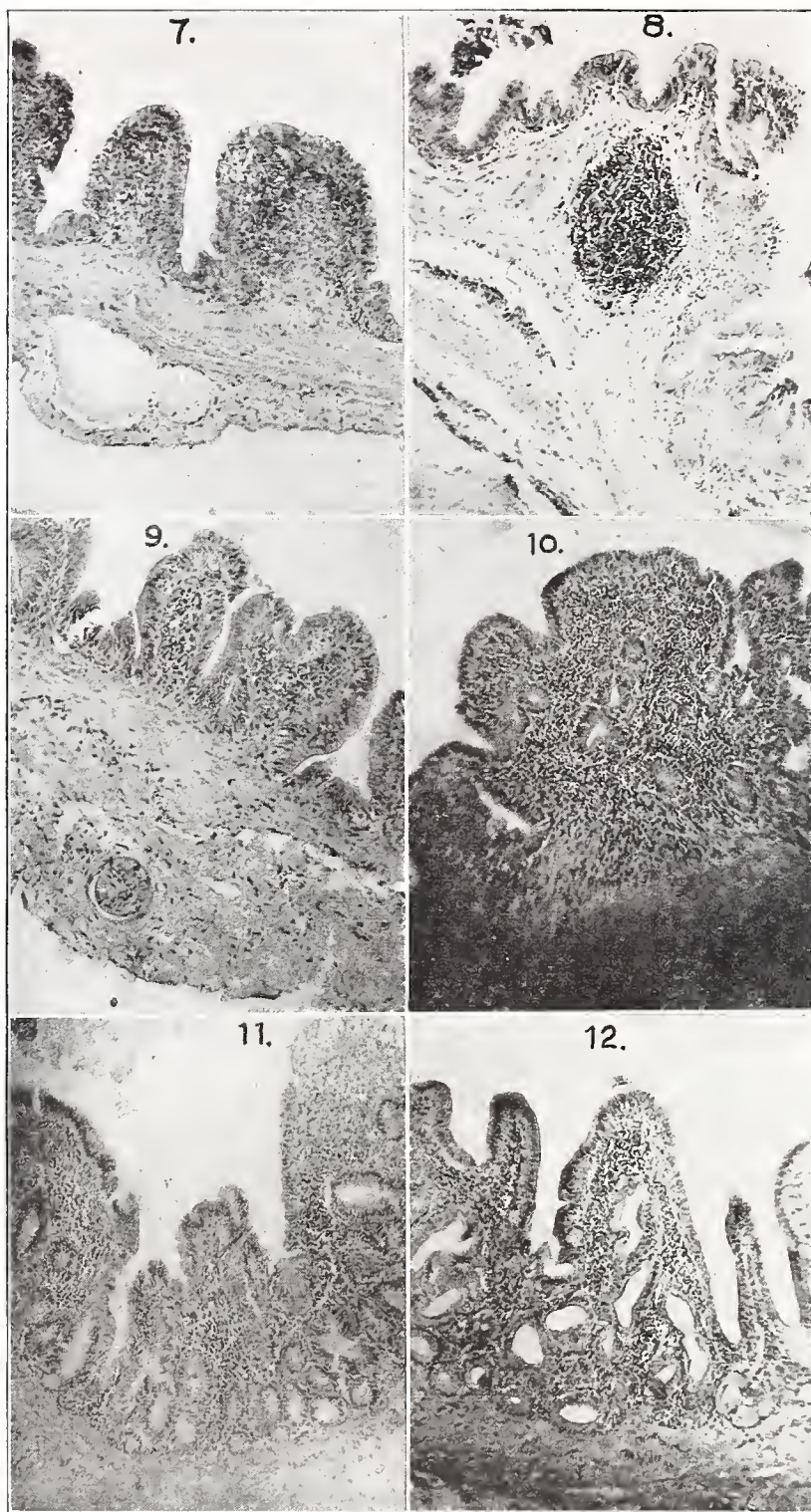


Fig. 7.—Rabbit 1021, 7 days, immunized, 10 billion agglutinated *B. typhosus* intravenously; gallbladder distended by colorless slimy pus, no erosions; extensive edema along extrahepatic ducts.

Fig. 8.—Rabbit 1095, 8 days, polyhomogenous *B. typhosus*, gallbladder distended, yellowish nodules and diphtheritic patches on mucosa; bile limpid, colorless. *B. typhosus* in all the organs.

Fig. 9.—Rabbit 1082, 31 days, 3 injections *B. typhosus* of recent isolation; loss in weight; intermittent shedding; irregular thickening of gallbladder wall, bile viscid, light green. *B. typhosus* in all the organs except heart blood.

Fig. 10.—Rabbit 1072, 32 days, polyhomogenous mixture of *B. typhosus* I. V.; intermittent shedding, cholangitis, empyema of distended thick walled gallbladder; *B. typhosus* in bile and wall, liver and duodenum.

Fig. 11.—Rabbit 705, 46 days; paratyphosus A; stool continuously negative; contracted gallbladder with 4 greenish soft stones size of a large pinhead; greenish watery bile. *B. typhosus* in bile and middle lobe of liver, duodenum.

Fig. 12.—Rabbit 15 A, 149 days, polyhomogenous I. V.; intermittent intestinal discharge of typhoid bacilli; gallbladder distended, 5 c.c of light green bile with biliary sand about $\frac{1}{3}$ of fluid. *B. typhosus* in bile and urine.

by way of the lymphatics; the resulting reactions involve these systems and not the blood vessels. Culturally, the bile invariably contains more typhoid bacilli than the gallbladder wall.

In studying this diffuse reaction it has been noted that the microscopic changes are the same whether the cystic bile has been infected by a direct injection or whether the bacilli enter the bile by way of the hemato-hepatogenous route and multiply in the bile. Moreover, the diffuse inflammatory reaction which follows the transverse embolic wall infection is of the same character. On the 3rd to 8th day after an intravenous injection of a large number of typhoid bacilli it is usually impossible to state from the study of a few sections whether the bacilli reach the gallbladder through the bile or through the blood vessels of the wall. In either case the bile in the viscus has become, on account of the addition of pus, lymph and blood fluids, an excellent substratum for the multiplication of the typhoid bacillus and invariably contains more germs per 1 c c than the wall. Their presence reacts on the layers of the wall in a manner absolutely identical with the one described for the intracystic injection. These facts explain in part the reports of Weinfurther,⁵⁸ Nichols¹⁰⁷ and others, who failed to observe the necroses, the diphtheritic inflammation of the villi, and the mechanism of the blood vessel injury which lead to the cholecystitis on account of their failure to make their histologic examinations sufficiently early.

The cellular reactions which develop in the course of the infection of the gallbladder wall are variable; they are usually diffuse, but may occasionally be localized in form of mural abscesses. The importance of these lesions on the persistence of the infection will be treated in a subsequent paragraph, but attention must be called at this point to a section of a gallbladder which was removed from a rabbit on the 30th day after infection (fig. 9). The connective tissue in the submucosa is increased, the blood vessel walls are either thickened and infiltrated with small lymphocytes or they exhibit organizing thrombi, all evidences of what is apparently a chronic inflammatory process. The origin of these extensive blood vessel lesions and their ultimate fate are difficult to determine in that we have not been afforded the opportunity of studying them at varying periods of their development. A systematic search will probably produce additional evidence to support the fact that the infection of the gallbladder wall plays an important rôle not only in streptococcic (Rosenow⁶⁷), but also in typhoid and paratyphoid cholecystitis.

The typhoid bacillus reaches the gallbladder in human cases of typhoid regularly after the liver has been disabled by the poisonous products of the bacteria, and its presence there is evidenced by positive cultures or by a mild or moderately severe catarrhal cholecystitis (E. Fränkel,¹⁰⁸ Posselt¹⁰⁹). According to the findings of numerous pathologists, the lesions present in the gallbladder and liver are certainly not as severe as frequently assumed by bacteriologists and epidemiologists. Purulent, necrotizing, inflammatory processes are infrequent and the formation of typhoid foci in the gallbladder wall supposedly of hematogenous origin are exceptionally rare. A general application of the conclusions of J. Koch¹¹⁰ based on an exceedingly severe and unique form of typhoid cholecystitis is by no means justified.

From our own limited number of observations¹¹¹ and the evidence which we have been able to gather from the literature (Bindseil; Messerschmidt¹¹²; Goebel; Nichols, Simmons and Stimmel¹¹³; Küster, and Günzel¹¹⁴), it is evident that the embolic infection of the gallbladder wall plays an insignificant rôle in the human carrier state. A condition analogous to that found in man apparently exists in the guinea-pig. The microscopic and cultural study of a limited number of gallbladders derived from infected guinea-pigs convinces us that the wall is rarely, and then only slightly, infected through the bile. In fact, properly immunized guinea-pigs never develop gallbladder infections.

The factors responsible for the hematogenous infection of the gallbladder of the rabbit have not been studied with the desired thoroughness on account of the large number of animals required for such tests. It has been stated in connection with our work on paratyphoid B infections in these animals, that intravenous or intraportal injections are prerequisites for the development of a cholecystitis. Feeding or subcutaneous injection fails to cause an infection. Similar observations were made by Fränkel and Much⁶⁹ with a specifically elective para-

¹⁰⁸ Mit. a. d. Grenzgeb. d. Med. u. Chir., 1909, 20, p. 898.

¹⁰⁹ Ergebn. d. allg. Path. u. path. Anat., 1919, 19, pp. 351 and 471.

¹¹⁰ Ztschr. f. Hyg. u. Infektionskrankh., 1908-09, 62, p. 1.

¹¹¹ We examined several gallbladders of convalescent typhoid patients which had been removed on account of gallstones. Histologically a diffuse infiltration of the mucosa and submucosa, or nests of round cells covered by an intact epithelium were observed. The cultural findings in one case also support the anatomic findings that the wall is not seriously involved, namely, the bile contained 30,000,000 typhoid bacilli per c.c. while 1 gm. of washed wall contained 240 bacilli.

¹¹² Ztschr. f. Hyg. u. Infektionskrankh., 1913, 75, p. 411.

¹¹³ Jour. Am. Med. Assn., 1919, 73, p. 680.

¹¹⁴ Ztschr. f. Hyg. u. Infektionskrankh., 1916, 81, p. 447.

typhoid bacillus. In experimental typhoid the individual susceptibility of the rabbits has interfered seriously in the numerous tests undertaken to determine some of the factors which lead to a wall infection. Typhoid strains which produce extensive liver necroses, provoke, as a rule, similar lesions in the gallbladder, provided the inoculated dose of bacteria is sufficiently large and made by intravenous, arterial or intrasplenic injections. Agglutinated bacteria or those grown on potatoes or blood agar give positive results more frequently than those cultivated in broth. Furthermore, starvation and changes in diet appear in some of the experimental series to be the factors conducive to wall infections.

THE FACTORS RESPONSIBLE FOR THE PERSISTENCE OF THE TYPHOID BACILLUS

Some writers on experimental cholecystitis conclude from their studies that the gallbladder is the only site in which the typhoid bacillus persists long after it has disappeared from all other parts of the body. These conclusions are based on a limited number of experimental animals and on incomplete tissue cultures. The recent reports of Hailer and Ungermann,¹⁰⁵ of Wagner and Emmerich,¹¹⁵ fail to support this view. The first named writers found the spleen infected in 56%, the liver in 66%, and the kidneys in 46%; while the gallbladder or its contents harbored the typhoid bacillus in 93% of the instances. The data of Wagner and Emmerich demonstrate even more conclusively the participation of other organs as sites for the persistence of the bacilli in the rabbit. Animals killed on the 226th or 346th day harbored typhoid organisms in the spleen, lungs, liver and gallbladder. We collected at random the necropsy findings of 18 rabbits, which were killed or had succumbed to an intercurrent infection on the 64th to the 816th day after an intravenous inoculation. These animals were chosen because minutely detailed cultures were made on their tissues. The findings are shown in table 4. Additional data will be published in subsequent papers.

It is evident from table 4 that animals which succumb to intercurrent infections (due to *B. cuniculisepticus*, enteritis) or perhaps auto-infections, may exhibit an extensive dissemination of typhoid bacilli in the various viscera. Rabbits, on the other hand, which were in good health when killed, usually showed typhoid bacilli only in

¹¹⁵ Med. Klin., 1916, 12, p. 819.

TABLE 4
ANATOMIC AND CULTURAL FINDINGS IN CHRONIC TYPHOID INFECTIONS OF RABBITS

Rabbit	When Killed After Infection	Anatomic Findings on Gallbladder	Bacteriologic Findings												
			Bile	Gall-bladder Wall	Liver	Spleen	Bone-marrow	Kidneys	Urine	Mesenteric Lymph Nodes	Lungs	Heart Blood	Duodenum	Jejunum	Ileum
840	180 days	Small, thick wall, bile light green with granular calculi.....	T	T	0	0	0	0	0	0	...	0	0	0	0
884	81 days	Distended, thick, light blue, granular sediment about 1/2 calculi	T	T	0	0	T	0	0	0	...	0	T	T	T
893	245 days	Distended, cystic duct blocked, light green with blackish calculi	T	T	T	0	T	0	0	0	T	0	0	0	0
10	99 days	Distended; thick wall infected, light green bile with coarse granular sediment	T	T	T	0	0	0	0	0	0	0	T	T	T
13	103 days	Distended, very thick wall, light green bile with coarse granular sediment	T	T	0	0	0	0	0	0	0	0	0	0	0
19	103 days D. pneumonia and pericarditis	Distended, very thick wall, purulent whitish with sediment....	T	T	T	T	T	T	T	—	T	T	—	T	T
800	83 days	Typical thick wall and light green bile, heavy sand-like debris	T	T	T	0	0	0	0	0	T	...	T	T	T
805	84 days	Distended, thick wall, light green bile with pus and sandy debris	T	T	0	0	0	0	0	0	...	T	0	T	T
836	232 days	Thick wall, adhesions, light green bile with blackish calculi..	T	T	T	0	0	0	0	0	0	0	T	T	T
865	409 days	Thick wall, light green, sand-like sediment with blackish calculi.	T	T	0	0	0	0	0	0	0	0	T	T	T
912	64 days	Thick wall, purulent flocculent bile, yellowish concretions, extensive cholangitis	T	T	T	T	T	T	0	0	0	T	0	0	0
918	pneumonia	Thick wall, light greenish bile, sand-like debris.....	T	T	T	0	0	0	0	0	0	0	T	0	T
740 days		Thick wall, adhesions, pure pus, and blackish debris, cystic duct obstructed	T	T	T	0	0	0	0	0	0	0	0	0	0
102 days		Thick wall, yellow, slimy purulent bile.....	T	T	T	0	0	T	0	0	0	0	0	0	0
1634	249 days	Thick wall, clear light green bile, cholangitic abscesses.....	T	T	T	0	0	0	0	0	0	0	T	T	T
946	86 days	Thin walled, dark green.....	T	0	0	0	0	0	0	0	0	0	0	0	0
1667	162 days	Small, thick wall, few drops of light yellow-greenish sediment;	0	0	0	0	0	T	0	0	0	0	0	0	0
1636	251 days	viscus atrophic, cholangitis of the extra hepatic ducts.....	T	T	0	0	0	0	0	0	0	0	T	T	T
717	816 days (relapse ?)	Size of a plum, thick wall, blackish granules in purulent light brown (largest gallbladder noted).....	T	T	T	T	T	0	T	T	T	T	T	T	T
Para A.															

the liver, bone-marrow and intestinal content. The persistence of the microbes is therefore not confined to the bile and gall-bladder wall. In one exceptional instance an animal came to necropsy in which the injected organisms were found only in the liver; and in another isolated animal killed on the 162nd day, only in the bone-marrow. Under special conditions, a prolonged localization in the kidneys can be demonstrated. In guinea-pigs a general dissemination and persistence in the tissues is the rule, while gallbladder lesions may be absent although typhoid bacilli are continuously discharged in the stools.

Particular study has been given to rabbits which show foci in the liver and bone-marrow. Thus far typhoid bacilli have not been found in the intestinal tract, bile or gallbladder of animals which carried the infection essentially in the remote viscera. Even liver foci have been recognized culturally, while typhoid bacilli have been absent in the cultures made from the bile and the intestines. In our experience the intestinal discharge of typhoid bacilli in the rabbit is, as a rule, associated with a gallbladder infection. It remains, therefore, to be proved, at least for this animal, that a closed liver focus or any distant focus, provided gallbladder infection is absent, is in a position to infect the intestinal tract through the bile, to which the organisms are supposed to have been carried by the blood or lymph stream. The factors responsible for the persistence of typhoid bacilli in the rabbit are in all probability confined to the gallbladder and to the extra- and intra-hepatic biliary system.

The question, "What is the average period of persistence of typhoid bacilli in the gallbladders of rabbits and guinea-pigs infected by way of the blood stream or by direct injection," must be answered before an analysis of the causes can be undertaken. Most observers who have kept rabbits for periods extending over 30 days agree that the localization of the bacteria is a temporary one in 60 to 70% of the animals. Lemierre and Abrami⁴⁷ have noted a restitution of the inflammatory process on the 6th day, while Lentz, Hailer and Wolf⁶⁰ have observed recoveries at the end of the second and third week. Morgan⁵⁰ found at the end of the 4th month 3 out of 7 animals, and Doerr 1 out of 2 rabbits, free from typhoid bacilli. According to Hailer and his associates,¹¹⁶ the duration of the infection depended on the degree of the lesions; in the course of 2 to 3 weeks, 40% of the rabbits lost their infection. On direct inoculation into the gallbladder they failed in

¹¹⁶ Arb. a. d. k. Gsndhtsamte, 1914-15, 48, p. 80.

several rabbits to recover the organisms on the 32nd and 38th days. Similar findings were made by Emmerich and Wagner.¹¹⁷ Our observations on a series of 131 successfully infected rabbits showed that 85% gave positive cultures on the 26th to 34th day when the bile samples were obtained by gallbladder punctures; 10% of the animals exhibited thickened gallbladder walls, while 5% were macroscopically normal. Infected animals when kept under observation for 80 to 100 days recovered from the specific cholecystitis in 72% of the instances. After a lapse of from 120 to 450 days a series of 35 rabbits showed typical thickened gallbladder walls, but the typhoid bacillus was isolated only from 10 to 27% of the animals. The cultures of several gallbladders gave a growth of indifferent streptococci. Based on these data, it must be concluded that successful typhoidal gallbladder infection in rabbits is frequently a temporary process. Chronic infections comparable with those of the human carrier at the end of 3 months are found only in 10% of the infected rabbits. As a rule, direct intracystic inoculations are conducive to a prolonged persistence (for more than 100 days) in a higher percentage of cases than intravenous injections. This corresponds to the findings of Wagner and Emmerich. Rabbits with chronic infections are always in perfect health and gain in weight. They are occasionally the victims of intercurrent infections, but not to a greater extent than control animals.

It is obvious that these observations in rabbits are in many respects analogous to those in human carriers. It may be mere coincidence that the percentage of 11.6% of typhoid carriers found in India by Semple and Creig¹¹⁸ corresponds rather closely with the data collected on rabbits. The majority of the rabbits can, following the classification of Sacquépée,¹¹⁹ be placed in the group of "temporary" or "convalescent" carriers; while 10% develop into "chronic" carriers. It is a well-known fact that typhoid bacilli may persist in the stools of human beings for 3 months after apparent recovery from typhoid or paratyphoid fever, and according to Reibmayr¹²⁰ 25% of these convalescent carriers free themselves of the infection spontaneously. Moreover, Goubau¹²¹ has shown that autovaccination clears such carriers of the bacteria much sooner than when they are left untreated.

¹¹⁷ *Centralbl. f. allg. Path. u. path. Anat.*, 1916, 27, p. 433; *Med. Klin.*, 1916, 12, p. 74.

¹¹⁸ Scientific memoir by officers of the Medical and Sanitary Department of the Government of India No. 32, Calcutta, 1908.

¹¹⁹ *Bull. Inst. Pasteur*, 1910, 8, pp. 1 and 49.

¹²⁰ *München. med. Wchnschr.*, 1918, 65, p. 670.

¹²¹ *Arch. Méd. Belges*, Paris, 1917, 70, p. 590.

It must be borne in mind by every experimenter that spontaneous recovery of the temporary carrier state in rabbits is rather the rule than the exception. These animals are therefore unsuitable for practical chemotherapeutic tests. Chronic carriers, particularly rabbits, infected by direct inoculation of the gallbladder, develop lesions which cannot be treated pharmaceutically and usually present a therapeutic difficulty which is rarely encountered in human cases. Appreciating these limitations, it seems obvious that therapeutic results obtained in rabbits should not be unreservedly applied in drawing analyses as to the possibility of treating similar conditions existing in man. The proper procedure in studying the pharmacologic influence of various substances on typhoid carriers consists in testing them first on dogs with biliary fistulas, followed by experiments on chronic rabbit-carriers infected by the intravenous route. Then human cases, on whom a cholecystotomy has been done and drainage instituted, should be investigated in order that the bile secretion can be tested chemically as well as bacteriologically. Finally the same problems should be studied in chronic human carriers. In all typhoid cases an attempt should be made to prevent by proper treatment (high calory diet instead of starvation, which favors biliary stasis) the development of a carrier state. Carriers should be treated in the earliest possible stage by intensive autovaccination. For this purpose a few chemicals have been suggested, but their value has not been definitely proved. A prolonged carrier state usually leads to severe lesions which cannot be influenced by such procedures. The radical removal of the gallbladder is then not only from a hygienic standpoint, but also on account of the frequent presence of, or tendency toward, gallstones, in the interest of the carrier himself.

Having recognized in the rabbit the occurrence of temporary and chronic gallbladder infections, it is important to determine, if possible, the causes responsible for these variations. In this connection the various routes leading to gallbladder infections deserve further attention. A hemato-hepatogenous elimination of the bacteria is suspected of causing a temporary cholecystitis, while a wall invasion results in a chronic persistent infection. It has been repeatedly stated that the inoculation of several billion typhoid bacilli is necessary to produce an experimental gallbladder infection. In case this particular prerequisite is not fulfilled, neither a discharge of bacilli through the bile nor an embolic invasion of the wall takes place. It is known from our own observations and those published by others, that the bacterial

elimination in the bile is temporary and, as a rule, in the first 6 hours comparatively few typhoid bacilli reach the bile in the gallbladder, where unhindered multiplication can ensue. The few organisms which enter the bile are probably rapidly discharged into the intestines, as is evidenced by the findings in spontaneously and artificially produced (feeding) paratyphoid B infections (Litch and Meyer⁴³). In case focal lesions, which communicate with the biliary passages, are established, as can be proved by intrahepatic injections (Hailer and Rimpau, personal observations), the elimination of large numbers of typhoid bacilli may be continuous for several days. Injury of the liver is accompanied by intoxication, with numerous physiologic and metabolic disturbances, one of which is biliary stasis. The bacilli reaching this viscus may remain, therefore, a sufficiently long period to permit of multiplication in a medium excellently suited for this purpose. In turn, the inflammatory reaction of the mucous membrane contributes an admixture of cells and lymph material, which favors the growth of the organisms in the cystic bile. Anatomically a catarrhal cholecystitis with little or no involvement of the submucosa and muscularis may be recognized. In these cases cultures of the bile give innumerable colonies of *B. typhosus*, while the washed wall is nearly sterile. This development of a mild gallbladder infection can be readily studied by direct inoculation of a small number of typhoid bacilli into the gallbladder. The degree of inflammation is moderate and the process involves mainly the subepithelial lymph system of the mucosa with a temporary lymphostasis in the adjacent systems of the muscularis and subserosa (fig. 7). Rabbits harboring such gallbladders are in the group of temporary carriers. At necropsy on the 10th to 20th day one finds a normal sized gallbladder with a slightly thickened wall which appears rather light in color. The bile is light or deep green, limpid, but contains no concretions of mucus. The P^+_{H} of the bile is less than 7.2 and changes rapidly to P^+_{H} 8.0-8.6. Cultures give typhoid colonies varying in number from 10 to several hundred per loop of bile. A careful histologic study of a large number of such gallbladders invariably shows an intact epithelium with lymphocytic infiltration of the mucosa, moderate proliferation of the connective tissue of the submucosa and a well preserved muscularis; no primary wall infection can be detected. The anatomic evidence supports the contention that these types of subacute cholecystitis may heal in from 2 to 4 weeks and then terminate the carrier state.

The etiology of the prolonged, temporary (50 to 100 days) or the chronic carrier state cannot be determined with certainty from our material. However, certain factors conducive to the persistence of the infection and the chronicity of the process can in a number of cases be definitely recognized. Diphtheritic foci of the gallbladder mucosa discharging clusters of typhoid bacilli into the bile are usually associated with liver lesions. The bile is being seeded from these numerous extensive foci. The inflammatory reaction resulting from this heavy and continuous infection is most severe as can be seen in gallbladder specimens removed on the 6th to 10th day of the disease. In exceptional instances the wall may show deep-seated abscesses (fig. 7). Usually the mucosa is partially necrotic, covered with leukocytes and fibrin; the submucosa and serosa are infiltrated with cells and fibrin and may even show small and large hemorrhages. The intrahepatic bile ducts not infrequently exhibit a marked inflammatory reaction. The bile is purulent, quite often blood tinged. These extensive changes may lead to adhesions or in exceptional cases to perforation. It is quite evident that such a profound involvement of the biliary system produced various anatomic and physiologic conditions, which favor the continuation of the infection. In chronic rabbit gallbladder carriers one of two findings is quite constant, namely, (1) empyema of the viscus with severe inflammation of the wall or (2) biliary sand or small calculi. The macroscopic findings of a number of gallbladders are reported in table 4. Histologic studies have been made on fresh tissue. Specimens with denuded mucosa are of questionable value and have not been used. The main findings are briefly as follows:

The outstanding features of the microscopic picture of the gallbladder showing an empyema is the thickening of the mucosa and submucosa to 4 to 8 times its normal dimension. The papillae of the mucosa are stumpy and diffusely infiltrated with lymphocytes. Occasionally nodular areas resembling lymph follicles can be observed. The covering epithelium exhibits a marked hyperplasia, but is intact. The cellular infiltration extends to the muscularis, which is either atrophic or its oblique fibers are increased in numbers (fig. 10). The stroma of the fibrous serous coat is enormously thickened and permeated with nests of lymphocytes and leukocytes. Frequently the wall of the blood vessels is thickened and infiltrated. The inflammatory process involves the cystic duct and frequently the extra- and intra-hepatic biliary system. A distinct cholangitis is found in 50% of the rabbits

killed between the 50th and 100th day of the infection. The bile is stringy, purulent and colorless. On standing, leukocytes and some biliary sand are sedimented (from 1/2 to 2/3 of the fluid bulk).

The gallbladder of the animals examined after the 30th day of the infection and that of every rabbit which harbored the typhoid bacilli for more than 100 days exhibit additional noteworthy microscopic changes. The thickness of the wall is not materially increased, but the mucosa exhibits elongated proliferations of the papillae. The entire mucosa coat contains numerous glands, which frequently show papillomatous extensions into the muscularis and submucosa resembling an adenomatous growth. Hypersecretion of mucus is distinctly visible, (figs. 11 and 12). An intact but hyperplastic epithelium covers a diffusely cellular-infiltrated stroma. The connective tissue growth in the serous and even the muscular coat is extensive; the lymph cell infiltration is comparatively slight. Round cells are, however, frequent in the hepatic connective tissue. The bile is rather viscid, the P^+_{H} is above 7.8, contains either a large amount of coarse, sand-like biliary concretion or even one or several well formed faceted stones. The latter may be black and friable. The findings are characteristic of those found in chronic cholecystitis. Invariably cultures prepared from the bile, stones, and wall give abundant colonies of *B. typhosus*. It has been impossible to demonstrate conclusively by tinctorial methods the bacilli in the tissues.

About 40% of our carriers killed before the 100th day exhibited the lesions of an empyema of the gallbladder, which explains readily the persistence of the bacteria in the biliary system. The empyema develops in consequence of an obstruction of the cystic duct or from a loss of contractibility of the wall. The latter may follow or precede the obstruction and is probably caused by the extensive proliferation of the connective tissue, submerging the important longitudinal and oblique muscle fibers. According to the available notes, cultures from the duodenum are frequently positive. Evidently the bacteria are eliminated with the hepatic duct bile from certain intrahepatic foci, because the inflammation in the cystic duct is sometimes so profound as to prevent a discharge of the gallbladder contents. These hepatic areas of necrosis or the microcholangitic abscesses are probably analogous to the lesions described by Blachstein,⁷ and they explain the occasional persistence of typhoid bacilli in the stools of rabbits after cholecystectomy which does not always cure intestinal carriers (Loeie¹²²; in exper. 1 and 2).

Exper. 1.—Rabbit 1625, weighing 2,175 gm. was inoculated on Aug. 8 and 9, 1918, with 1 mg. of uranium nitrate, subcutaneously. On Aug. 20, 1918, it received an injection of 1,500 million *B. typhosus*, grown on potatoes, directly into the left kidney. The stool and urine were positive on Aug. 29, Sept. 2, 11, 15, 23 and Oct. 1. On Oct. 3 a complete cholecystectomy of an enlarged typical gallbladder was performed. The stool was negative on Oct. 4, 5, 6, 8, 9, 15, 25 and Nov. 10; while the urine was positive on Oct. 4, 5 and 8, and negative on Oct. 9, 15, 25, 30, and Nov. 10. At necropsy, Dec. 24, 1919, all the organs were found sterile.

Exper. 2.—Rabbit 1630, weighing 2,175 gm., was inoculated Oct. 8 and 9 with 1 mg. of uranium nitrate, subcutaneously. On Aug. 20, 1918, an injection of 1,500 million typhoid bacilli grown on potatoes was made directly into the left kidney. The urine was positive on Aug. 23, 29, Sept. 8, and Oct. 1. The stool contained bacilli on Aug. 29, Sept. 8, and Oct. 1. A complete cholecystectomy was performed on Oct. 3, 1918. The gallbladder was typical; the bile contained 3,200,000 bacteria per c.c. and the wall 3,500,000 per 100 mg. Recovery from the operation was rapid and complete. Urine cultures remained negative, while the stool cultures were positive on Oct. 4, 5, 6, 8, 15, 22, 28, 30, Nov. 10, and then remained negative on daily examination. At necropsy on Dec. 21, or 78 days after the operation, *B. typhosus* was isolated from the liver, the left kidney, the left bone marrow and a small abscess between the stomach and the liver. The intestinal tract was negative for *B. typhosus*.

From these experiments it is evident that the removal of the gallbladder does not always cure the carrier state, although the observations are not sufficient to warrant absolute deductions. In this respect the findings are analogous to those reported for human carriers, namely, cholecystectomy does not always cure intestinal carriers (Loele,¹²² Fromme;¹²³ Schultze;¹²⁴ Nichols, Simmons and Stimmel¹⁰⁷).

The bile of "chronic carriers," that is, rabbits which remained infected 100 to 816 days, contained invariably yellow-greenish biliary concretions, or blackish calculi. It appears of interest to consider briefly the nature and origin of these stones. The small concretions are microchemically composed of lime salts, traces of bile pigment and organic material, but no cholesterol. They are "Entzündungsteine" in the sense of Aschoff and Bacmeister,¹²⁵ or calcium concretions, which develop as a result of the inflammatory process. It is well known and has been repeatedly shown that a chronic cholecystitis leads to an hyperplasia of the mucous glands and to an excessive production of biliary mucus. Aschoff believes this mucus to be very rich in lime salts. Lichtwitz and Bock¹²⁶ consider the calcium content of the bile

¹²² Deutsch. med. Wchnschr., 1909, 35, p. 1429.

¹²³ Deutsch. Ztschr. f. Chir. 1910, 107, p. 578.

¹²⁴ Centralbl. f. d. Grenzgeb. d. Med. u. Chir., 1913, 49, p. 1892.

¹²⁵ Die Cholelithiasis, Jena, 1909.

¹²⁶ Deutsch. med. Wchnschr., 1915, 41, p. 1215.

to be normal. Its precipitation, however, is facilitated by the excessive and concentrated glandular secretion.

Irrespective of the uncertainty relative to the origin and the physical-chemical factors which lead to the precipitation of the calcium salts of the bile, it has been definitely proved (Rosenbloom¹²⁷) that biliary calculi composed of lime are regularly due to an infectious process. The conditions favorable for the development of such stones are unquestionably present in the chronic cholecystitis of the rabbit. Cal-

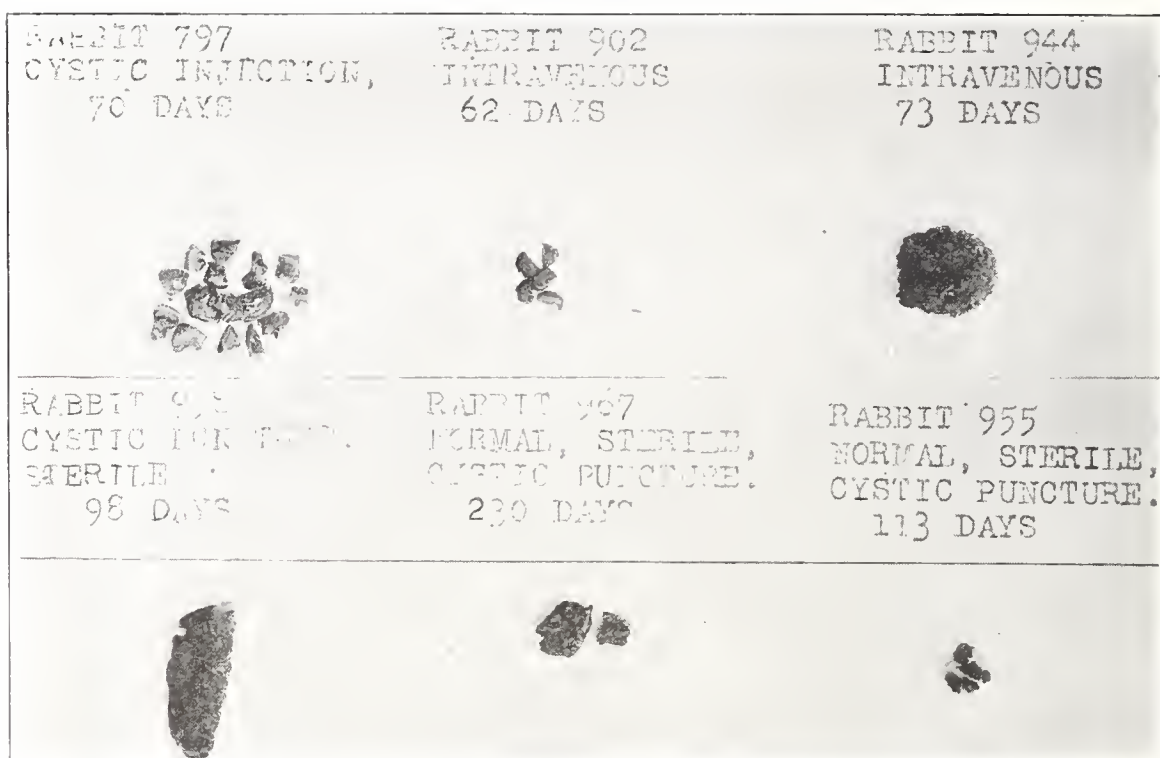


Fig. 13.—Stones (actual size) originating in gallbladders of rabbits inoculated intravenously with typhoid bacilli.

culi once formed favor the perpetuation of the inflammatory process and with it the persistence of the typhoid bacillus. The precipitated calcium salts are in part also responsible in carrier rabbits for the well-known low H-ion concentration of the cystic bile exposed to air for a few minutes. The large black or greenish stones have, in our opinion, a different origin. They are soft and friable; chemically they are composed of protein material (30 to 60%), some bile pigment and at times

¹²⁷ Jour. Am. Med. Assn., 1917, 69, p. 1765.

traces of lime salts. Certain stones (example shown, natural size, in fig. 13) originate in the gallbladders of rabbits which have been inoculated intravenously with typhoid bacilli. They are likewise found in this viscus after a direct inoculation by puncture of the gallbladder. It has been noted that blood clots develop quite frequently subsequent to a needle puncture or the application of a ligature. Hemorrhages and blood extravasations also result, in intravenously inoculated rabbits, as the consequence of the diphtheritic, necrotizing cholecystitis. The albuminous, organic nucleus of these stones is therefore primarily one of these blood coagulums, which in some cases may secondarily become impregnated with calcium salts (fig. 13, rabbit 944). It is obvious, that absorption of this type of stone is practically impossible and their presence assists in the prolongation of the cholecystitis and the persistence of the infection. This interpretation probably applies to the frequent findings of biliary calculi of this type in the gallbladders of rabbits infected by direct cystic injections (Emmerich and Wagner).

The concretions ordinarily found in rabbits should not be compared with the cholesterol stones of man. This fact has been emphasized by Klinkert,¹⁸ who repeated the experiments of Cushing,¹²⁸ in which the gallbladder was first injured by compression with forceps, and the animal subsequently infected by an intravenous injection of typhoid bacilli. Bilirubin-calcium calculi could be found on the 54th day after the operation. Pure cholesterol stones artificially placed in the gallbladders of rabbits are, in our experience, gradually dissolved. By feeding cholesterol or lanolin we succeeded in producing pure cholesterol calculi, as will be discussed in paper VI of this series. Biliary calculi have been observed occasionally in rabbits infected with typhoid or colon bacilli and cholera vibrios (Richardson¹ Cushing;¹²⁸ Doerr;¹ Forster and Kayser;⁴⁶ Creig⁸⁴). Spontaneous stone formation without previous bacterial injury of the gallbladder wall has not been reported. The absence of calculi in the rabbits killed before the 30th day (our own observations are supported by those of Hailer and Rimpau) and their constant presence in animals necropsied after the 100th day are significant. It justifies the conclusions that the chronic inflammation provoked by the typhoid bacillus is the cause of the precipitation of the lime salts. The resulting calculi in turn are probably the main factors favoring the persistence of the bacteria in the gallbladder.

¹²⁸ Bull. Johns Hopkins Hosp., 1899, 10, p. 166.

These findings indicate that the persistence of the typhoid bacilli in the rabbit's gallbladder is quite analogous to the carrier state in man. This latter condition is, according to the observations of Nichols, Simmons, and Stimmel,¹¹³ of Messerschmidt,¹¹² of O. Mayer,¹²⁹ and others, associated in 65 to 90% of the cases with gallstones, while the remaining 10 to 35% of human carriers invariably show a distinct chronic cholecystitis or an empyema of the gallbladder. The excellent summaries of Posselt¹⁰⁹ and of Wagner and Emmerich¹⁵ supply additional information for this comparison and should be consulted by those interested in the subject of cholecystitis caused by typhoid fever.

In the preceding discussion the function of the bile has not been considered as a factor controlling either the development or the persistence of a carrier state in rabbits. Comparatively recently Nichols expressed the opinion based on test-tube experiments that the biles of certain rabbits possess an inhibitory or even an antiseptic action. Our detailed studies reported in paper VI, VII and VIII, fail to support this contention. In fact, it is proved that the cystic bile of rabbits is particularly suited for the growth of *B. typhosus*.

TABLE 5
AGGLUTINATION OF TYPHOID BACILLUS IN CARRIERS AND RECOVERED CARRIERS

Carrier		Recovered Carriers	
99 days.....	1:1,000	134 days.....	1:2,000
103 days.....	1:1,000	149 days.....	1:2,000
140 days.....	1:2,000	180 days.....	1:2,000
180 days.....	1:2,000	205 days.....	1:600
232 days.....	1:600	252 days.....	1:100
245 days.....	1:4,000	314 days.....	1:600
409 days.....	1:400	430 days.....	1:200

A comparative analysis of a limited number of serologic tests fails to suggest any relation between demonstrable immune bodies and the carrier state. This should be expected in the light of the data discussed in paper IV. The serum of chronic carrier animals agglutinates the typhoid bacillus in dilutions above 1:100 quite frequently in dilutions as high as 1:4,000. In comparison with the original titer determined 15 to 20 days after the infection, the agglutinating power of the serum is considerably diminished. After a lapse of 100 to 200 days the immune bodies remain, however, fairly constant. Recovered carriers differ in no respect from the animals harboring a focus of infection, as is illustrated by the figures in table 6. Complement fixing antibodies

¹²⁹ München. med. Wchnschr., 1914, 61, p. 1116.

disappear more rapidly than the agglutinins, but no differences are noted between the infected and the recovered animals. It is well known that in rabbits the disappearance of agglutinins following an infection is fairly rapid in the first 2 to 3 months, and may remain constant for over one year. Guinea-pigs behave differently; such animals harboring typhoid bacilli in their tissues may give negative Widal reactions on the 50th or 60th day after infection. It is evident that serum tests can neither be used to detect a carrier state in rabbits or guinea-pigs, nor can they explain the recovery or the persistence of the bacilli in the tissues and particularly the gallbladder. Experiments conducted several years ago have proved that cutaneous hypersensitiveness tests are more marked in infected than immunized rabbits. Skin tests may therefore be employed to advantage in the detection of the rabbit carrier state.

In conclusion, it can be stated that the temporary typhoid carrier state in the rabbit is probably the result of a hemato-hepatogenous infection of the bile. The resulting inflammation of the gallbladder is comparatively mild and a *restitutio ad integrum* can take place readily. The chronic carrier state is favored by a severe wall infection resulting from an embolic invasion of the terminal capillaries of the mucosa. The persistence of the typhoid bacillus is facilitated by an obstruction of the cystic duct, the development of an empyema and the formation of biliary calculi. These chronic infections can be produced more readily in rabbits resistant to the typhoid toxin or by the injection of an atoxic strain in doses of not less than 2 billion per kilogram.

GALLBLADDER INFECTIONS PRODUCED BY THE INOCULATION OF
TYPHOID BACILLI INTO THE SPLEEN AND INTO THE
GALLBLADDER CONTAINING GALLSTONES

Hailer and Ungermann¹⁰⁵ attempted by direct inoculation of *B. typhosus* into the liver, kidneys, duodenum, appendix and gallbladder to produce typhoid foci in which the bacilli were protected from the destructive influence of the blood serum (?). It was also expected that these areas would constantly reinfect the other tissues of the body and favor a more chronic infection than could be obtained by intravenous injections. With the exception of the direct inoculations into the gallbladder and perhaps the liver and kidney, neither of the procedures offered any advantage over the usual intravenous method. The injections into the intestinal tract gave inconstant results, while the liver injections provoked extensive necroses which had nothing in common

with the lesions found in man. Our experience with these methods was similar.

The explanation of the carrier state by Webb-Johnson⁸ already referred to, suggested the advantage of direct inoculation of typhoid bacilli into the spleen. This is, however, not the only reason why this method has been tried. There are many arguments which may be advanced against the intravenous injections. In the first place, there is necessarily considerable dilution of the infective material and an extremely good opportunity for the operation of any deleterious action which the plasma and cells may possess. Moreover, it seems logical to introduce the infective agent into the locations which are involved in the spontaneous infections as exemplified in the experimental production of tuberculosis and syphilis by inoculation into the testes. The spleen is readily exposed by laparotomy under ether anesthesia, and subcapsular injections of 0.5 to 1.0 c c can be made without injury to the organ.

Thus far we have practiced this method on 16 large rabbits (average weight 2,240 gm.). They have all recovered from the operation; when killed 11 to 31 days after the injection, 8 animals harbored typhoid bacilli in the gallbladder, spleen, liver, bone-marrow and intestines. The intoxication has been slight in comparison to that of the intravenously injected animals. In this connection it should be emphasized that the number of typhoid bacilli introduced did not exceed 500 million. It is well known that this dosage of bacilli produces gallbladder lesions only in exceptional instances in small rabbits. The remaining 8 rabbits are still under observation and are apparently infected, judging from the presence of the typhoid bacilli in the stools. The local lesions produced by the infection are slight; a few adhesions of the splenic capsule to the peritoneal wall, a minor spleen tumor and a small area of necrosis are the only changes recorded on the 31st day. From an experimental standpoint this method is an excellent one and a high percentage of carriers with a low initial mortality can be produced. These results naturally incline one to accept the view of Webb-Johnson. The spleen of the human carrier may be the focus which continuously supplies the bile with the specific organisms. Typhoid bacilli have been found in the spleen of the majority of chronic human carriers, examined after death. Their presence has been attributed to auto-infection, but some of the patients unquestionably died from pneumonia, apoplexy (Günther and

Böttcher,¹³⁰ Kamm¹³¹), without symptoms of auto-infection. In rabbits, however, a chronic gallbladder infection has never been found to be associated with a splenic focus. In our experience the only positive spleen cultures obtained were made from animals which died from intercurrent diseases (table 4, 19, 64, and 816). It is, however, possible that the direct splenic inoculation, which reproduces the conditions in man more accurately than the intravenous method, may furnish conclusive support to the above theory. Such information can, however, only be procured from animals which have been kept under observation for several years. A final opinion concerning this interesting conception of the carrier state by Webb-Johnson will be rendered when the studies of the remaining 8 rabbits are completed.

There had been considerable discussion as to whether the typhoid bacillus is capable of exciting the formation of gallstones or whether the stones are usually preformed, and as such predisposed to a localization of the invading organism. Having satisfactorily demonstrated the lithogenic properties of the typhoid bacillus in the gallbladder of rabbits, it is of interest to test experimentally the second view. The experiments conducted for this purpose are not entirely satisfactory. The introduction of a stone invariably leads to a secondary invasion of the gallbladder wall or the bile by "indifferent" intestinal streptococci. Under these circumstances the viscus is predisposed to a subsequent infection and typhoid bacilli as well as streptococci thrive in the tissues. In some experiments the typhoid bacillus is isolated from the gallbladder wall, while the bile itself contains streptococci. Moreover, it is proved that a relatively small dose of bacteria may localize in the gallbladder in which a calculus has been previously placed. Two experiments of a series of 8 are presented below to illustrate the above statements.

Exper. 1.—Rabbit 1399, weighing 3,095 gm., was laparotomized on March 3, 1919, and a fragment of a sterilized cholesterol stone removed from a human typhoid carrier was placed in the gallbladder. On June 9, 1919, or 97 days after the operation, the animal was killed. The gallbladder was distended and filled with pus-like yellowish fluid containing small fragments of the inserted stone and fine sand-like débris. The wall was thick and fibrous, the mucous coat was injected and partially denuded. The serous coat was edematous and the periportal lymph nodes were soft and edematous. The other organs were normal. *Streptococcus salivarius* was isolated from the bile, gallbladder wall, regional lymph node and duodenum.

¹³⁰ Ztschr. f. Hyg. u. Infektionskrankh., 1911, 68, p. 439.

¹³¹ München. med. Wchnschr., 1909, 56, p. 1011.

Exper. 2.—Rabbit 1323, weighing 3,675 gm.; on Nov. 8, 1918, a sterile stone the size of grain was placed in the gallbladder. On Dec. 27, 1918, the animal weighed 3,750 gm., and was injected intravenously with $\frac{1}{100}$ slant or 400,000,000 typhoid bacilli. On Jan. 20, 1919, the rabbit had diarrhea and appeared sick. It was chloroformed. At necropsy extensive adhesions between the liver, stomach and omentum were found. The gallbladder wall was thick, and contained about 3 cc of turbid, viscid, brownish-yellow bile. The stone was partially disintegrated and pigmented. The mucosa was smooth; no erosions were visible. Spleen and lymph nodes were enlarged, and soft; a moderate catarrhal enteritis was present. The bile contained streptococci, while the gallbladder wall, the liver, right kidney, spleen and lung gave pure cultures of *B. typhosus*. The streptococcus grew in opaque, grayish-white staphylococcus-like colonies.

From these observations it becomes evident that streptococci regularly invade a gallbladder injured by an operative manipulation or when irritated by a calculus. The origin of these bacteria is probably intestinal and the route is a lymphogenous one. A subsequent typhoid bacillus invasion is unable to dislodge these streptococci, but fosters a prolonged sojourn and wide dissemination of *B. typhosus* in the body. According to Rosenow,⁶⁷ Brown,¹³² Starr and Graham,¹³³ and others, streptococci are frequently found in acute and subacute cholecystitis of man. The foregoing data suggest a method to reproduce these infections experimentally. In our experience streptococci may replace a colon or typhoid infection in the gallbladder of a rabbit; it is therefore recommended that extreme caution be exercised in the interpretation of the presence of streptococci as an apparent selective affinity for the gallbladder. In order that this type of experiment may be of some clinical value it is advisable to extend the period of observation over several years. It is not unlikely that such studies may assist materially in an understanding of gallbladder disease of man.

SUMMARY AND CONCLUSIONS

The elimination of typhoid bacilli in the hepatic duct bile of normal and immunized animals has been studied on rabbits, guinea-pigs and dogs provided with temporary common duct fistulas. These experiments have demonstrated that more bacteria appear in the bile of normal rabbits inoculated intravenously with 8,000 to 24,000 million typhoid bacilli than in that of immunized animals of the same litter, provided the last inoculation of the vaccine is administered 20 to 30 days previous to the infection.

¹³² Arch. Int. Med., 1919, 23, p. 185.

¹³³ Ann. Surg., 1918, 68, p. 188.

The elimination of the bacteria by the hemato-hepatogenous or descending route is immediate. The maximum number of colonies develop on the plates prepared with bile specimens collected between the first 5 to 15 minutes following the injection. In subsequent periods the number decreases rapidly and the discharge of bacteria may cease completely at the end of one hour. The ability of the individual rabbit to eliminate bacilli in the bile varies considerably. Even repeated inoculations of large doses (10 to 15 billion) may in a small number of rabbits never lead to a discharge of typhoid bacilli in the hepatic duct bile.

The transit of the bacilli from the hepatic blood vessels to the biliary capillaries is probably governed by the phagocytic action of the endothelial cells. Immunization of the rabbits prevents, to a certain degree, the passage of bacilli. In the immunized guinea-pig the endothelial barrier is exceedingly efficient and an intravenous injection of less than 100 million typhoid bacilli usually gives sterile bile cultures, while in normal guinea-pigs several hundred organisms are eliminated in the fistular bile following similar injections.

Rabbits which have been inoculated with dead or living typhoid bacilli and which have been injected intravenously with living typhoid bacilli on the 6th to 10th day after the last immunizing injection, discharge more bacilli than the normal control animals of the same litter. This exceptional behavior of the immunized rabbit is probably due to an incomplete *restitutio ad integrum* of the injured vascular endothelium. Complete recovery from bacterial vaccination, which evidently does not take place before the 10th day, as a rule prevents the transit of bacilli from the blood to the bile.

The excretion of typhoid bacilli in the hepatic duct bile of dogs is irregular and not definitely influenced by immunization. Injections of less than 10 billion bacilli in dogs varying in weight from 14 to 26 pounds fail to cause an elimination of bacteria.

The removal of bacteria from the circulation in the first 10 to 60 minutes may be the result of an *in vivo* agglutination, the action of the blood platelets, or purely a dispersion phenomenon of two colloids. Virulent paratyphoid B bacilli of rabbit origin are clumped as readily and disappear as rapidly from the blood as the noninvasive typhoid bacilli. The removal of bacteria from the peripheral circulation is decidedly more rapid in immunized than in normal rabbits and guinea-pigs.

The leukopenia of the peripheral blood following the injection of bacilli is the result of an uneven distribution of the leukocytes which are chemotactically attracted by the bacillary masses collecting in the viscera.

Histologic evidence is presented which indicates that the gallbladders of about one third of the rabbits injected with large doses of typhoid bacilli receive the infection through the terminal capillaries of the mucosa. Preparations made from gallbladders removed on the 24th to 72nd hour after the injection show necroses and diphtheritic inflammatory areas in the villi of the mucosa. The transverse route of bile infection through the wall occurs also after ligation of the cystic duct.

Direct gallbladder injections produce an infection of the wall along the lymphatic system of the mucosa, submucosa and subserosa. The epithelium remains intact and focal necroses of the villi are not observed. In case the typhoid bacilli reach the gallbladder by the hemato-hepatogenous route only, they multiply in the cystic bile, which is suitable for their development. The histologic changes produced in the wall are identical with those provoked by direct inoculation.

Serial sections of the fundus portion of the gallbladder wall removed on the 3rd to 5th day following the infection may indicate on histologic examination the route responsible for the gallbladder lesion, whether descending or transverse.

Rabbit typhoid carriers may be classified into temporary or convalescent and chronic carriers. Thirty to 40% of the intravenously inoculated animals recover from their infection in the first month after the injection. About 10 to 15% may retain typical bacilli in the gallbladder for 6 months to one year, occasionally even longer. The persistence of the micro-organisms depends on the degree of inflammation provoked in the gallbladder. In case the bacillary invasion of the bile is innocuous to the tissues or produces only slight catarrhal cholecystitis, a temporary infection will be noted. It is not unlikely that such a state is regularly the result in descending hemato-hepatogenous infections.

Chronic carriers result probably from embolic, capillary invasion of the wall, with subsequent transverse infection of the bile. Persistence of the bacteria is favored by the formation of biliary calculi (60 to 80% of the cases), by the extension of the inflammatory process to the cystic ducts, and by a severe cholecystitis leading to a loss of contractibility of the wall, followed by a state of empyema. The biliary

calculi consist usually of bilirubin-calcium material. The nucleus of this may be an unabsorbed blood clot. Cholesterol stones have not been found. Chronic gallbladder carriers frequently harbor typhoid bacilli in the liver, bone-marrow, lungs and intestines. In exceptional instances isolated foci of infection may be found in the bone-marrow and kidneys. The elimination of typhoid bacilli in the feces of rabbits is, however, practically always associated with gallbladder or bile passage infections. It remains, therefore, to be proved that a close liver focus or any distant focus, provided the gallbladder infection is absent, is in a position to infect the intestinal tract through the bile, to which the organisms are supposed to have been carried by the blood or lymph stream. This contention is sound, notwithstanding the fact that in a few instances it has been demonstrated experimentally that in certain rabbits with infected gallbladders and extrahepatic biliary passages, cholecystectomy does not prevent the elimination of typhoid bacilli in the stools.

Intrasplenic injections of typhoid bacilli produce regularly persistent gallbladder infection. The conception of the human carrier state advanced by Webb-Johnson is discussed.

The insertion of sterile gallstones into the gallbladder of rabbits leads to a secondary infection of this viscus by indifferent streptococci. Such gallbladders are predisposed to a localization of the typhoid bacilli introduced by way of the blood streams.

The typhoid bacillus reaches the gallbladder in human cases of typhoid fever regularly after the liver has been disabled by the poisonous product of the bacteria, and its presence therein is evidenced by positive cultures or by a mild or moderately severe catarrhal cholecystitis. According to the findings of numerous pathologists, the lesions present in the liver and gallbladder are certainly not as severe as is frequently assumed by bacteriologists and epidemiologists. Cholecystitis is a far more frequent complication during typhoid fever than is generally supposed, but purulent necrotizing inflammatory processes are not common, and the formation of bacillary foci in the gallbladder wall supposedly of hematogenous origin is exceptionally rare. Application of the conclusion of J. Koch based on an exceedingly severe and unique form of typhoid cholecystitis is by no means justified.

THE REACTION AND PHYSIOLOGY OF THE HEPATIC DUCT AND CYSTIC BILE OF VARIOUS LABORATORY ANIMALS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VI

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In the preceding papers we have shown that typhoid bacilli are frequently discharged in the bile of practically every animal that has been inoculated intravenously with large doses of bacteria of the typhoid-paratyphoid group. Accepting the statements and interpretations of others, it became quite evident that the biliary secretions were under suspicion as important factors in the development of carriers.

Since Nichols issued his studies on the supposedly antiseptic effect of rabbit bile, we were compelled to verify the published facts and to correlate the findings of others with our own. In this connection we studied primarily the antiseptic action of animal and human bile. As Nichols¹ considered the latter to be largely due to its alkalinity, we measured the H-ion concentration of biles derived from a large number of rabbits, guinea-pigs, dogs, rats, cats, etc. We found rather constant difference in the reaction between the bile derived from the liver by a common duct fistula and the one obtained from the gallbladder. We noted that the former became more alkaline on standing outside of the viscus, a final H-ion concentration above P_H 9.0 being frequently reached. Most of our determinations were made on animals that had been kept on controlled-diets, and the bile specimens were obtained by temporary common ducts fistulas. In the course of the collection of these samples a number of physiologic data which are rarely mentioned in the literature were recorded. They contribute important information to the physiology of biliary secretions and to the function of the gallbladder, and are therefore included in this paper.

No exact data as to the H-ion concentration of the hepatic duct and gallbladder bile of rabbits, guinea-pigs, rats, monkeys and man were available. Preliminary tests were made by the titration method, but we were convinced that the titrable acidity or alkalinity did not repre-

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¹ J. Exper. Med., 1916, 24, p. 497.

sent the true H-ion concentration of the bile specimen. In measuring the H-ion concentration it was also recognized that special precaution had to be observed in order that the escape of CO_2 did not decrease measurably the H-ion exponent. As stated in the introduction, we have also compared, where possible, the reaction and physiology of gallbladder bile with the hepatic duct bile. In a limited number of experiments we tested the influence of diet on biles, the biles from carrier animals, and followed the changes of bile samples, which had been neutralized in vitro.

METHODS

The hepatic duct bile samples were collected from temporary common duct fistulas. The technical procedures employed have been detailed in the preceding paper.

Cystic bile was procured at necropsies or by laparotomies, a capillary Pasteur pipet being inserted through the seared gallbladder wall and the contents aspirated. In normal healthy animals invariably sterile bile was present. Our observations fail to support the claims of Ehret and Stolz,² who recorded that 11 of 17 guinea-pigs' bile tested were contaminated with various organisms. Gallbladder bile of large, domesticated animals was obtained from the slaughter house. The isolated cystic duct was tied and the whole bladder wrapped in gauze soaked with bichloride solution. In the laboratory the bile was aspirated by means of a Pasteur pipet, which was introduced through a cauterized area of the bladder wall. In contrast to Ehret and Stolz, Létienne³ and others, the samples obtained in this manner were sterile in 90% of the instances.

In studying the reaction of the bile specimens we had access to two methods: the titration method and the colorimetric method. The titration method was handled by us thus: One part of bile diluted in 9 parts of freshly boiled and cooled neutral distilled water mixed with phenolphthalein (0.1 c c of a 2% solution in 50% alcohol to 10 c c of diluted bile solution) was titrated with the aid of a micro-burette (0.1 c c pipet) with a decinormal solution of a sodium hydroxide or hydrochloric acid as the case may be. The end-point arbitrarily chosen by us was the first perceptible change in color recorded during the first minute. The solutions of hepatic duct bile were always clear, and a slight change was readily noted in contrast with the control. Heavily pigmented or turbid

² Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 7, p. 372.

³ Arch. de méd. expér. et d'anat. path., 1891, 1, p. 761.

cystic biles of man, monkey, dog, etc., were more difficult to titrate. The first discernible change in color covers a broader range and consequently may easily increase the experimental error. Individual differences of interpretation were eliminated by the same person always making the readings.

We have deviated from the methods of Jolles ⁴ and others in slightly varying the amount of indicator used rather than the diluent. For example, we have uniformly used 0.1 c c of phenolphthalein solution for amounts of 0.1 c c to 1 c c of bile. And again in dark and turbid biles we have found it simpler and more expeditious to double or even to quadruple the quantity of indicator rather than to increase the amount of diluent. The comparatively small amounts of bile aspirated from the gallbladder of guinea-pigs and rabbits that was to be titrated before cultivating forced us to titrate only 0.1 c c lots of bile, in which instances the reaction was determined with N/50 solutions of alkali and acid. For measuring the alkalinity of bile samples sensitive azolitmin indicator solution ⁵ was used in the same manner. However, for the sake of economy in titrations an approximate range of the reaction of biles was first obtained by indicator papers or solutions.

For reasons to be discussed, the colorimetric method for the H-ion determination was adapted for the study of the reaction of bile samples. It is generally accepted that only the accurate determination of the actual hydroxyl-ions should be used to express the reaction of mediums or biologic fluids. Before the publication of Clark and Lubs ⁶ dealing with a dependable procedure had appeared, and a set of brilliant indicators had been found, we followed the outline described by Hurwitz, Meyer and Ostenberg.⁷ It is quite natural that the method of Clark and Lubs, which covers a broader range of reaction, should have replaced the obsolete procedure adopted at the beginning. This in part will explain why some of our results dealing with P_{H^+} determinations are expressed as >8.6 and >9.2 .

The reaction of the bile is progressively changing and therefore the electrometric method, being time-consuming, would favor erroneous results. However, for old biles exposed to various factors for several days, the electrometric method should be used more frequently. Unfortunately, we were not in possession of an equipment to make such read-

⁴ Arch. f. d. ges. Physiol., 1894, 67, p. 1.

⁵ Sørensen: Ztschr. f. phys. Chemie, 1909, 64, p. 120.

⁶ J. Bacteriol., 1917, 2, pp. 1, 109 and 191.

⁷ Johns Hopkins Hosp. Bull., 1917, 2, p. 2.

ings when necessary, but through the courtesy of Dr. Schmidt, several samples were checked electrometrically, and the P_{H^+} readings were found to correspond closely with those obtained by the colorimetric method. For biologic work, minor differences of one or two degrees are of considerably less importance than in accurate chemical work. We have recently tested simultaneously a number of old hepatic duct bile samples of the rabbit by the electrometric and colorimetric method and have convinced ourselves that the readings made by the latter are absolutely reliable.

As already suggested in the introduction, it was our purpose to determine the reaction of biles, hepatic duct or cystic, *in vitro* with the least exposure to disturbing factors. It is therefore a great advantage to note from the paper of Clark and Lubs⁸ that the "dilution method" is with safety applicable to the H-ion determination of turbid fluids. They established the legitimate dilution as 1:5, but they also mentioned that highly colored solutions may be diluted to a point where they may be used in the comparator. Furthermore, they showed⁹ that the 1:10 dilution is reliable for approximate determinations upon a wide variety of solutions highly colored and turbid (p. 193). We have found that a dilution of 1:10 influences the final P_{H^+} readings slightly. It is possible to check this conclusion on lightly colored biles (hepatic duct of guinea-pigs, dogs, etc.) in an undiluted state. A sufficiently large series of comparative tests with highly colored biles has convinced us that a dilution of 1:10 shows approximately the same P_{H^+} values as the one of 1:5 and has also been proved by electrometric determinations.

It has been emphasized that small amounts of bile are in this study the only available material. The dilution method is the only feasible procedure by which we were able to obtain comparable data on the behavior of hepatic duct and cystic bile of one and the same animal. Therefore we have adopted for colorimetric determinations of all biles a 1:10 dilution (0.2 c c bile in 1.8 c c freshly boiled and cooled distilled water mixed with 0.2 c c of indicator). Invariably the P_{H^+} determinations were made with two or more indicators, which cover our ranges, namely: brom thymol blue, phenol red, cresol red and thymol blue. It is always advisable to use at least two indicators overlapping the probable range of the H-ion concentration of the bile. Shades of color which develop under varying CO_2 tensions and which make comparison

⁸ Footnote 6, p. 129.

⁹ Footnote 6, p. 191.

TABLE 1
TITRABLE ACIDITY AND ALKALINITY OF VARIOUS BILE SAMPLES

Animal	Diet	Method of Obtaining Bile	Gallbladder Bile				Hepatic Duct Bile					
			Number of Biles Tested	Titration of Phenolphthalein	Degree of Acidity	Titration of Phenolphthalein	Degree of Alkalinity	Number of Biles Tested	Titration of Acidity	Degree of Acidity	Titration of Alkalinity	Degree of Alkalinity
Rabbit	Mixed	Chloroformed	15	0.8 (0.1-2.7)	0.032	0	0	7	0.13 (4)* (0.1-0.2)	0.52	0.18 (5) (0.1-0.2)	0.064
	Immunized	Or laparotomy										
	Immunized	After death	6	3.5 (2.0-6.4)	1.40	0	0	7	0.14 (5) (0.01-0.2)	0.18 (3) (0.1-0.3)	0.064
	Immunized carriers	Laparotomy	18	0.2 (3) (0.1-0.4)	0.08	0.39 (ampho-theric -1.1) (15)	0.1404
	Immunized carriers	After death	3	1.5 (0.5-4.0)	0.60	0	0
	Oats	Laparotomy	3	0.46 (0.4-0.6)	0.184	0	0	7	0.06 (2)	0.024	0.15 (5)	0.054
	Wheat	Laparotomy	2	1.12	0.448	0	0	2	0.06 (2)	0.024	0.05 (1)	0.018
	Cabbage	Laparotomy	6	0.61	0.244	0	0	9	0.01 (5)	0.004	0.29 (7)	0.104
	Carrots	Laparotomy	2	0.65	0.260	0	0	2	Ampho-theric (1)	0.05 (1)	0.018
	Cholestearolemia	Laparotomy	1	0.2	0.08	0	0	3	0	0	0.13 (3)	0.046
	Alkalosis	Laparotomy	3	0.57	0.228	0	0	4	0.3 (1)	0.12	0.16 (3)	0.057
	Sodium bicarbonate	Laparotomy	3	0.77 (0.1-2.5)	0.308	0	0	4	0.27 (3)	0.18	0.2 (1)	0.072
	Acidosis with HCl	Laparotomy	3	0.77 (0.1-2.5)	0.308	0	0	4	0.27 (3)	0.18	0.2 (1)	0.072
	Starvation	Laparotomy	2	1.32	0.528	0	0	2	0.15 (1)	0.06	0.2 (1)	0.072
Guinea-pigs..	Mixed	Laparotomy	47	0	0	0.43 (0.1-1.5)	0.1548	6	0	0	0.55 (6)	0.198
	Mixed	Laparotomy	33	2	0.1 (1)	0.04	0.3 (1)	0.108
	Mixed	Laparotomy and after death	1.15	0.460	3	0.15-0.2	0.06
	Mixed	Laparotomy	2	1.6	0.64	2	0.2	0.08
	Laparotomy and after death	7	1.6	0.64	1	0.75-1.0	0.3
	December	2 hours after slaughtering	8	0.55	0.220
	December	2 hours after slaughtering	9	1.9	0.76
	December	2 hours after slaughtering	2	0.75	0.30	1	0.35	0.14
	December	2 hours after slaughtering	10	1.05	0.42
	Biliary fistula	Immediately	1	0.1-0.2	0.04-0.05
Man.....	Sodium bicarbonate and Carlsbader salt	2.2 to haemoid
	Light diet	Cholecystectomy	2	0.8 (1.0-0.6)	0.32

* Numbers in brackets refer to the number of animals tested.

difficult with one indicator, frequently can be matched by another, for example, thymol blue is superior to cresol red.

Pyrex glassware was used in all titration procedures. At the beginning of our studies the bile specimens were collected in open 15 c.c. graduated centrifuge tubes and when filled a colorimetric determination was immediately made. In order to avoid exposure of the bile to the factors changing the reaction, we resorted to the aspiration of the flowing hepatic duct bile by means of a hypodermic "Record" or any tightly fitting syringe. In the course of our inquiry, we finally developed a procedure that accomplishes a similar result in a more convenient manner, namely, the bile is collected directly under a 2 to 3 cm. layer of paraffin oil.

Cystic biles were removed by means of a hypodermic syringe or a Pasteur pipet and transferred to stoppered vials. They were either immediately titrated and tested or placed under oil for a short time.

To avoid a misunderstanding or repetition, we desire to specify that bile specimens standing in test tubes covered with or without oil for from 6 to 10 hours at room temperature or in the ice-chest and stoppered loosely by cotton or cork are considered "old biles."

RESULTS WITH RABBIT BILE

We examined 64 gallbladder and 55 hepatic duct biles of rabbits by the titration and by the colorimetric method. More than 100 gallbladder biles were tested by the colorimetric method only. The results dealing with the titrable acidity and alkalinity are shown in table 1. With the exception of the samples of cystic bile derived from "carriers," which will be discussed separately, all samples were definitely acid to phenolphthalein. The degree of acidity varied from 0.032 to 1.40 mg. of NaOH necessary to neutralize 1 gm. of bile. Rabbits fed on a mixed hay, oats and cabbage diet showed gallbladder bile of lowest acidity; those kept on wheat or starved, or fed with HCl, produced cystic biles that were distinctly acid. The most pronounced acid reaction was noted for gallbladder biles of rabbits which were not necropsied for at least from 5 to 10 hours.

The reaction of the hepatic duct bile of the same rabbits was either amphoteric, very slightly acid, or of various gradations from slightly to decidedly alkaline. About one-half of the specimens immediately titrated were alkaline to phenolphthalein, in contrast to the constant acid reaction of the gallbladder biles. The base-forming diets, particularly cabbage, produced hepatic duct biles, which were distinctly alkaline to phenolphthalein; 1 gm. of bile required 0.104 mg. of HCl for neutrali-

zation, using azolitmin as an indicator. Intravenous injections of NaCO_3 , or feeding of the same substance in combination with base forming diet, gave common duct bile samples which differed little from those obtained from rabbits kept on mixed feeds.

The phenomenon of gradual increase of the alkalinity on standing was also noted when using the titration method. For example, a bile specimen giving a titrable acidity of +0.25 may on standing become amphoteric to phenolphthalein in 24 hours. It is evident that this reaction of the hepatic duct bile is different from that of the gallbladder. As a rule the titrable reaction of the former is more variable and more inclined to the acid side. All hepatic bile specimens were distinctly alkaline to litmus, 23 (or 58%) of 40 gallbladder biles tinged red litmus paper blue, 7 (or 17.7%) were amphoteric and 10 (or 25%) merely changed it to yellow.

TABLE 2
H-ION CONCENTRATION OF GALLBLADDER BILE OF ANIMALS FED ON VARIOUS DIETS

	Number of Animals	P_{H^+}	Average P_{H^+}
Mixed diet.....	15	6.6-7.2	6.7
Mixed diet and fasting.....	10	5.8-6.8	6.4
Acid forming diet (oats).....	5	6.4-7.4	7.0
Experimental acidosis.....	3	6.4-6.8	6.6
Base forming diets.....	10	7.0-8.2	7.7
Experimental alkalosis.....	3	6.8-8.4	7.6

These differences became more apparent when we determined the H-ion concentration of the individual bile samples. Immediately after withdrawal from the gallbladder the bile showed an H-ion concentration expressed in a P_{H^+} ranging from 5.7-7.4. The average reaction varied between P_{H^+} 6.7-7.0, that is to say, the cystic bile of the rabbit was either acid or neutral or slightly alkaline. It may, however, become strongly alkaline on standing or give an initial P_{H^+} reading above 7.2 when the secretion has reached the gallbladder shortly before its removal.¹⁰ Only in exceptional instances did we notice after 24 hours' standing a reaction of less than P_{H^+} 8.6. The factors responsible for this alteration will be analyzed. This variability of the reaction of the gallbladder bile is apparently existent in different individuals, and was in our experience closely connected with the general health and the diet of the animals. The figures in table 2 will illustrate this statement.

¹⁰ The P_{H^+} readings of such specimens are recorded in the tables in brackets.

We were particularly interested in the determination of the factors influencing the H-ion concentration of the gallbladder bile and conducted a fairly large series of determinations. The figures noted must be considered strictly as averages; it is difficult to state with absolute certainty the origin of the fluid withdrawn from this organ. In animals with fistula it is not unlikely that hepatic duct bile has regurgitated into the bladder shortly before the sample has been removed and our determinations are made on liver instead of on bladder bile. For practical purposes, it is clear that mixed and acid-forming diets, including HCl are conducive to gallbladder biles which are slightly acid. This likewise is the case in fasting animals, in which there is apt to be stasis in the gallbladder. The reaction on standing remains either stationary or increases slowly. On the other hand, base-forming diets, such as cabbage and carrots and sodium bicarbonate feeding, produce alkaline cystic bile samples, which show a rapidly decreasing H-ion concentration on standing.

Hepatic duct bile, which is withdrawn from the collecting rubber tubing and immediately titrated, is always alkaline. Even when exposure to the air is reduced to a minimum, the P_{H^+} reading never goes below P_{H^+} 7.5, and in the majority of instances it is between P_{H^+} 8.2-8.8. It will be noted from table 2 that the change in H-ion concentration is very rapid and that a bile sample examined 2 hours or more after removal from the body may give a P_{H^+} reading from 8.4 to >8.6 . This change is absolutely constant and is not markedly influenced by the general health of the animal or its diet. We shall discuss our experiments, which were conducted in an endeavor to produce such differences, after we have considered the factors responsible for the progressive changes on standing.

MECHANISM OF THE CHANGES IN H-ION CONCENTRATION OCCURRING IN FISTULA BILE ON STANDING

That changes occur in the reaction of biologic fluids on standing is well known, but to our knowledge particular attention has not been called to this phenomenon in connection with the titration of bile samples. Recent controversies dealing with the H-ion concentration of cerebrospinal fluids, and the careful studies of Levinson¹¹ suggested that the changes occurring in the hepatic bile on standing resulted from similar causes. A priori we could readily eliminate the conception that the

¹¹ J. Infect. Dis., 1917, 21, p. 556; Cerebrospinal Fluid, 1919, p. 96.

change was due to alkalis given off by glassware. Only carefully tested nonsol or pyrex test tubes were employed in our colorimetric determination. In analogy with the findings of other workers on biologic fluids, it suggested itself that the changes taking place on standing were either the result of loss of CO_2 or the formation or absorption of alkaline substances like ammonia.

We tested these conceptions by the following experiments: hepatic duct bile collected by aspiration or under paraffin oil in a test tube was divided into 6 portions of from 5 to 10 c c. The reaction of these samples at the beginning of the experiments was P_{H^+} 7.8-8.0. Sample 1 was covered with oil and tightly sealed with a rubber stopper; sample 2 was similarly closed, but without oil. Samples 3 and 4 were stoppered, leaving a small air space between the bile and the cork or rubber stopper. Sample 5 was placed in a test tube loosely closed by a cotton plug. Sample 6 was exposed in an open test tube in a desiccator to CO_2 and ammonia-free air. All samples were tested after 10 minutes, 1, 2 and 12 hours. The results of some of these experiments, which were repeated several times, are graphically summarized in chart I.

It may be concluded from these experiments (chart 1) that the H-ion concentration of hepatic duct bile decreases rapidly when the specimens are exposed to alkalis or to open air. The escape of CO_2 can be prevented or reduced by stratification with paraffin oil and tight sealing of the test tube with a cork or rubber stopper. The latter experiment also demonstrates that the decrease in the H-ion concentration is not the result of ammonia production in the fluid. In tubes tightly corked with air bubbles excluded, the bile either retained its original P_{H^+} for 12 to 24 hours or was only slightly above it (curves 1 and 2). On the other hand, exposure to NaOH or vacuum produced a rapid decrease of the H-ion concentration showing that accelerated loss of CO_2 is responsible for the increase of the P_{H^+} .

In a parallel set of experiments, hepatic duct bile collected in an open tube was distributed in lots of 5 c c each in test tubes, which were loosely plugged with cotton. The beginning H-ion concentration of the bile was P_{H^+} 8.2 (+0.35). Sample 1 was heated in an open water bath at 56 C. for 30 minutes in order to dispel quickly the CO_2 ; samples 2 and 3, both not heated, one stratified with paraffin oil and the other exposed to air, were placed with sample 1 in an open water bath at 37 C. Sample 4 was exposed in a chamber with free ammonia; sample 5 in an ammonia-free NaOH-desiccator, and sample 6 in a tube in which the air had been displaced by oxygen. These specimens were incubated at 37 C. P_{H^+} determinations were made on all specimens at 1, 2, 24 and 48-hour intervals (see chart 6, second paper).

It was noted that there is little difference in the H-ion concentration of hepatic duct bile when heated at 56 C. for 30 minutes or exposed to

air or to oxygen or to an ammonia-free atmosphere in a closed desiccator. However, the specimen exposed to ammonia vapors shows that bile is capable of absorbing it readily. The H-ion concentration was materially lowered. It is suggestive that while the bile may not readily form ammonia-like substances on standing, it may absorb substances like ammonia from the environment. This may in a measure explain the variability of the reaction of certain bile specimens when exposed to air or kept in a desiccator. It was frequently noted that one and the same specimen may show a low H-ion concentration (lowest electrometric reading on the 15th day P_{H^+} 9.214) when exposed to air, while the reaction of the secretion kept in a desiccator was not lowered in the same degree. In the first few days the desiccator tubes may, as a rule, give a slightly higher P_{H^+} reading than the air tube. However, this observation was frequently reversed and the air tube registered a lower H-ion concentration than the desiccator tube. We are under the impression that aside from the escape of the CO_2 another factor may influence the H-ion concentration. The fluctuating ammonia content of the laboratory atmosphere and the varying absorbability of this chemical by the bile may be responsible for the irregular P_{H^+} readings. Stratification with oil prevents the absorption of ammonia, but does not entirely inhibit the gradual escape of CO_2 . Moreover the P_{H^+} increase cannot be the result of an oxidation process. The H-ion concentration of the bile in the oxygen tube was not materially increased in contrast to the one kept under oil. However, what the actual influence of the ammonia absorption by the bile may be, the loss of CO_2 , whether rapid or slow, is in our opinion the main factor in the lowering of the H-ion concentration.

Exposure to air therefore produces an hepatic duct bile and, as already stated, also a gallbladder bile which differs materially from the one present in the rabbit body. We are dealing here with a distinct test-tube artefact. Two points deserve, however, a more detailed inquiry in the future, namely: 1. Is the decrease of the H-ion concentration always of the same rapidity in the hepatic duct and the gallbladder bile of individually different rabbits kept under identical conditions? 2. What is the final P_{H^+} of such biles when exposed to air for 24 hours? Thus far we possess only a few suggestive observations, namely, experimental alkalosis may produce hepatic duct biles in which the H-ion concentration decreases in a somewhat shorter time than in the secretion obtained from animals kept on acid forming or mixed diets. Aside

from the point already mentioned, we call attention to the slight fluctuations in the P_{H^+} of fistular bile in the course of a single experiment. In some instances the gradual increase in the H-ion concentration was mainly the result of frequent collections, which were made necessary by the administration of a cholagogue. On the other hand, a slight increase would be readily explained by the gradual CO_2 depletion of the blood and the disturbance of the alkaline reserve balance, which follows the operative procedures and the restriction of bodily movements unavoidable in these experiments. Most of our P_{H^+} determinations were made by means of the colorimetric method and represent therefore only relative values. For practical purposes it has been sufficiently proved that the bile of rabbits, irrespective of its origin, changes its reaction when standing exposed to air and that a P_{H^+} usually above P_H 8.4-9.0 may be reached in from 12 to 24 hours. The importance of the observation will be appreciated fully in the paper on the antiseptic effect of bile.

ALKALINE RESERVE OF HEPATIC DUCT BILE

We were able to determine the alkaline reserve as bicarbonate on the amount of CO_2 present in hepatic duct bile by the method of Van Slyke and Cullen.¹² It was impossible to obtain sufficient secretion from the gallbladder, which would have enabled us to study the alkaline reserve and the changes in the H-ion concentration simultaneously. Table 3 shows our findings.

TABLE 3
ALKALINE RESERVE IN HEPATIC DUCT BILE

	Blood Plasma, Volume %	Hepatic Bile Duct, Volume %
Rabbit 1418 (mixed diet).....	42.4	116.9 on standing
Rabbit 1801 (mixed diet).....	50.3	115.2 under oil 105.7 exposed to air

These data show that the alkaline reserve of hepatic duct bile is more than twice, nearly three times as great, as the one found in the plasma. The average is about 115 volume % of CO_2 found at 0 degrees temperature and 760 barometric pressure. Most of the CO_2 is apparently fixed in form of carbonates, but the additional presence of gaseous CO_2 , which escapes on standing, is shown by the lower volume in per-

¹² J. Biol. Chem., 1917, 30, p. 291.

centage finding of the specimen of rabbit 1801 when exposed to air. Our plasma bicarbonate figures are lower than those reported by Asada.¹³ The differences are probably explained by the fact that we used the heart blood of rabbits which had been operated on, and which had remained in a fixed position on the operation board for at least 6 hours; factors which are known to reduce the alkaline reserve of the blood.

CHANGES OF THE H-ION CONCENTRATION OF THE BILE IN EXPERIMENTAL CONDITIONS

Guided by Nichols's¹ conception that the germicidal property of rabbits' bile *in vitro* disappears on neutralization with a strong acid (HCl or H₂SO₄) and that therefore the reaction of this secretion governs its destructive influence on bacteria, we conducted tests to confirm the above claims. We furthermore attempted to support his second suggestion, namely, that rabbits in a state of alkalosis produced by a previous injection of sodium bicarbonate are protected against a gallbladder infection. Before one can accept this contention he must show that an experimental acidosis or alkalosis really influences the reaction of the bile. Okada¹⁴ failed to notice a change in the H-ion concentration of fistular bile of dogs fed 200 c c of N/10 HCl solution. On the other hand, we have already recorded the influence of base-forming diets on the biliary secretions of rabbits, a condition which we also attempted to enhance by the use of sodium bicarbonate. We attempted also to solve the next question, namely, is the reverse true? Does the feeding of HCl or a state of experimental acidosis increase the H-ion concentration *in vivo*? The latter procedure gave some suggestive results, but for practical purposes we can probably accomplish in the rabbit the same effect through fasting or the prolonged exclusive feeding of acid-forming diets. A few tests were also made on rabbits which were in a state of hypercholesterolemia.

EXPERIMENTS IN VITRO

Five to 10 c c samples of hepatic duct bile were carefully neutralized with undiluted lactic acid or N/10 HCl. The chemical was added drop by drop, the precipitate which formed under production of gas was redissolved by shaking the tube. A number of tests were also conducted with hepatic bile partially saturated with CO₂. P_H⁺ determinations were made at varying intervals.

¹³ Am. J. Physiol., 1919, 50, p. 1.

¹⁴ J. Physiol., 1915, 49, p. 457; 1915-1916, 50, p. 114.

TABLE 4
THE H-ION REACTION OF "CARRIER" BILES

Rabbit No.	Diet and Treatment	Agglutination Reaction of Bile	Serologic Findings of Blood Serum		pH ⁺	Appearance of Bile and Bacteriologic Findings
			Agglutination	Complement Fixation		
941	Mixed diet, laparotomy	1:10	1:1000	0.05 ++	7.2 (2') 8.4 (60')	Light greenish, limpid, ∞ B. typhosus
942	Mixed diet, laparotomy	>1:20,000	0.0005	9.0 (60')	Light green, limpid, thick wall, ∞ B. typhosus
943	Mixed diet, killed	8.0	Light green, limpid, thick wall, ∞ B. typhosus
944	Mixed diet, killed	1:6000	8.4	Light green with soft stone, ∞ B. typhosus
946	Mixed diet, killed	1:100	1:1000	0.05	8.2	Clear light green bile, wall thick ∞ B. typhosus
947	Mixed diet	<1:40	1:6000	0.005	8.0	Brownish slimy bile with brownish green debris, wall thick, ∞ B. typhosus
961	Mixed diet, laparotomy	<1:10	1:1000	>8.4 (30')	Light green, limpid, B. coli
963	Mixed diet, laparotomy	1:10	1:2000	0.05	8.4 (30')	Light greenish, yellowish slimy sediment, ∞ B. typhosus
671	Mixed diet, laparotomy	1:20	1:1000	>8.4 (30')	Light greenish bile, sediment, thick wall, B. coli
672	Mixed diet, laparotomy	1:400	0.005	8.3 (5')	Light greenish with adherent crusty sediment, ∞ B. typhosus
676	Mixed diet, laparotomy	1:800	1:1000	8.2	Purulent, colorless, viscid, wall thick
	Recovered at necropsy	1:20 +++	1:500	7.4	Clear yellowish green, sterile
997	Mixed diet, killed	1:1000++	7.3	Light green, considerable granular sediment, thick wall, ∞ B. para. B
1035	Mixed diet	8.0 (10')	Light greenish, limpid, ∞ B. typhosus
1048	Mixed diet, laparotomy	>1:1000	>1:20,000	<0.005	8.4 (30') (+0.1)	Light greenish, limpid, very little sediment, wall injected, ∞ B. typhosus
1053	Mixed diet, laparotomy	1:60	1:10,000	0.005	8.4 (30') (-0.1)	Colorless, sand-like yellow debris wall adhesions, ∞ B. typhosus
1054	Mixed diet, laparotomy	1:20	1:4000	0.01	8.4 (30')	Clear light green bile in large dilated gallbladder, ∞ B. typhosus
1056	Mixed diet, laparotomy	1:200	1:8000	0.005	8.2 (30')	Dark green viscid bile, sterile
1057	Mixed diet, laparotomy (coccidiosis, few patches)	1:200	>1:20,000	0.005	8.4 (30')	Purulent, colorless, slimy bile with considerable sediment, ∞ B. typhosus
1059	Mixed diet, laparotomy	1:20	1:6000+++	0.005	8.4	Light greenish bile with greenish sand-like sediment, wall adherent, abscesses, ∞ B. typhosus
1059b	Mixed diet, laparotomy	<1:10	1:1000	0.005	8.4	Light greenish limpid bile with considerable sand-like sediment, ∞ B. typhosus
1124	Oats, killed	1:20,000	7.5	Purulent, slimy with considerable sediment, wall thick, ∞ B. typhosus
1129	Oats, killed	7.0 (2')	Purulent, slimy with considerable sediment, wall thick, ∞ B. typhosus
1131	Oats, killed	1:10,000	7.5	Greenish, very slight sediment, ∞ B. typhosus
1135	Oats, killed	1:600	7.7 (5')	Purulent slimy, slightly colored, ∞ B. typhosus
1157	Oats and starvation	7.6	Purulent, colorless, sand-like, greenish sediment, ∞ B. typhosus
1160	Oats, severe infection	1:10-20	1:2000+++	7.5 (5')	Slimy, colorless, sand-like sediment, 11,800,000 B. typhosus per 1 c c
1163	Oats, Na ₂ CO ₃	<1:10	1:1000	7.8	Light green, limpid, sterile, staphylococcus 100 per c c
27 Rabbits	Average....	1:200 (1:10-1000)	1:400-1:20,000	8.0	

* If not specifically stated the reactions were determined immediately on collection.

From the data thus collected it became apparent, that neutralization changes only temporarily the H-ion concentration of hepatic duct bile when exposed to the air. In some biles the H-ion concentration decreases progressively in 24 hours until a reaction identical with the original specimen is reached. Preservation of bile under oil delayed this decrease and in most instances the original P_{H^+} was not attained. These observations, therefore, indicate that neutralization does not prevent a bile specimen from changing its H-ion concentration. Invariably such fluids exposed to the air reached a reaction which must be considered unfavorable for the growth of *B. typhosus* and other organisms.

In a few experiments hepatic duct bile of a P_{H^+} of >8.8 was partially saturated with CO_2 , which increased the H-ion concentration to P_{H^+} 6.8-7.0. Exposure to the air in a water bath caused a rapid decrease, and in 24 hours the original P_{H^+} was reached. Stratification with oil naturally retarded the return to a low H-ion concentration.

EXPERIMENTS IN VIVO

A small series of rabbits was kept on different diets. Common duct fistulas were made on these animals. The H-ion concentration of the blood was determined by the dialysis method of Levy, Rowntree and Marriot;¹⁵ the the alkaline reserve, R_{PH} , according to the procedure of Marriot; and the reaction of the urine by the colorimetric method. Collections of hepatic duct bile before and after the feeding of varying amounts of HCl or intravenous injection of $NaHCO_3$ were made, and the reactions determined.

In the course of these tests it became evident that final conclusions cannot be drawn from the limited number of experiments. In most instances striking changes in the H-ion concentration of the hepatic bile were not demonstrable. In only two rabbits (692 and 1118), which had been nourished on a mixed oat and hay or cabbage diet respectively for one month, there was recorded a slight decrease in the P_{H^+} of the hepatic duct bile during the 2 hour period following the administration of HCl by the stomach tube. In one animal (1118) the R_{PH} of the blood had fallen from 8.6 to 7.8 and the urine also turned acid. In another rabbit (1031 b) the H-ion concentration of the blood decreased following the use of HCl. The R_{PH} remained unaltered, and consequently the influence of the P_{H^+} of the hepatic bile was not felt in the two periods following the feeding of HCl, or was so slight that our crude methods failed to record it. We desire also to call attention to the fact that the literature records cases of experimental acidosis in rabbits, in

¹⁵ Arch. Int. Med., 1915, 16, p. 389.

TABLE 5
ACCORDING TO THE FOLLOWING AUTHORS ONE KILOGRAM OF ANIMAL WEIGHT SECRETES IN 24 HOURS

	Hepatic Duet Bile		Remarks
	Rabbit	Guinea-Pig	
Heidenhain (1883).....	136.84 c c	175.84 c c	Gallbladder fistulae (?)
Mann (1918).....	56.84 c c	130.29 c c	Hepatic duet fistulae
Neilson and Meyer (1920).....	90.34 c c (17 animals)	154.27 c c (9 animals)	Hepatic duet fistulae
Average.....	71.34 gm.	153.46 gm.	

TABLE 6
THE RELATION OF BILE SECRETION TO THE BODY AND LIVER WEIGHT OF RABBIT AND GUINEA-PIG

	Rabbit	Guinea-Pig
1. Average weight: Heidenhain.....	1525.8	518
Mann.....	2158.8	561.8
Neilson-Meyer.....	2506.6	899.2
2. Fresh bile per kg. body weight in one hour:		
Heidenhain.....	5.070	7.32
Mann.....	2.36	5.42
Neilson-Meyer.....	3.76	6.42
3. Proportion of liver weight to body weight:		
Heidenhain.....	1:33.5	1:27.3
Mann.....	1:30.3	1:18.1
Neilson-Meyer.....	1:31.1	1:22.09
4. Fresh bile per kg. of liver per 1 hour:		
Heidenhain.....	169.3	185.5
Mann.....	84.9	130.6
Neilson-Meyer.....	122.1	134.5

TABLE 7
THE INFLUENCE OF DIET ON THE RATE OF BILE FLOW PER KILOGRAM WEIGHT FOR RABBITS, GUINEA-PIGS AND RATS

Number of Animals Tested	Diet and Treatment	Average Weight, Gm.	Average Weight of Liver, Gm.	Average Amount of Bile Collected in the First Hour, C c	Average Amount of Bile Collected During 6 Hours, C c	Average Estimated Total of Bile for 24 Hours, C c	Average Capacity of Gall-bladder C c	Average Percentage of Bile Secreted in 24 Hours, Which the Gallbladder Will Hold
4 rabbits.....	Mixed	2,725	90.5	10.09	56.1	218.4 (80.1 per kg.)	3.1	1.4
5 rabbits.....	Mixed and cholagogue	2,385	73.1	11.9	42.5	156.1	2.0	1.5 (?)
2 rabbits.....	Fasting	1,990	52.0	5.65	33.7
4 rabbits.....	Oats	2,650	76.0	9.75	67.03	279.9 (105.62 per kg.)
7 rabbits.....	Cabbage	2,145	75.25	9.69	45.7	182.9 (85.26 per kg.)	1.8	0.96
2 rabbits.....	Carrots	1,982.5	62.0	10.4	51.75	223.5	2.0	0.7
2 rabbits.....	Oats and Na ₂ HCO ₃	2,925	112.0	12.0	64.2	256.8	2.8	1.2
9 guinea-pigs..	Mixed	899.2	40.7	10.95	138.82	1.5	0.96
2 rats.....	Mixed	298.5	0.97	1.3-5.1	23.28

which the changes of the buffer values of the blood are inconstant, and it is reasonable to suspect that unless the blood alkalies are seriously depleted no appreciable change will be noted in the reaction of the biliary secretions.

It is even more difficult to provoke changes by means of sodium bicarbonate in the H-ion concentration of the hepatic duct bile of rabbits fed with acid-forming diets. The alkali reserve can be readily increased, but in our few experiments this was not followed by any noticeable decrease in the H-ion concentration of the bile. Kuriyama and also McClendon, von Meysenberg and Engstrand¹⁷ noted the effect of diet on the alkaline reserve of the blood of rabbits, which is also indicated in our observation by the rather low R_{P_H} of several rabbits. The injection of 1 to 2 gm. of NaHCO_3 increased the R_{P_H} slightly, but the alkali depletion (acid diet, narcosis, etc.) was apparently so great that P_{H^+} of the urine was only slightly decreased. In one instance it was even increased. And again, in the oat fed rabbits, a marked holding back of alkalies took place. It may therefore be necessary to give more than 2 gm. of NaHCO_3 , just as is the case in pathologic conditions in order to produce their elimination with the urine or with the bile. The titrable alkalinity also showed no noteworthy increase. It should be recalled that only immediate determinations of the H-ion concentration will give P_{H^+} readings which are closely analogous to those of the blood. In a thoroughly alkalinized rabbit (1200) the rapid decrease in the H-ion concentration on standing, and the low final reaction has already been commented on and is undoubtedly the best proof that feeding or injection of alkalies may influence the reaction of bile. How far a moderate alkaline therapy may change the reaction of gallbladder bile cannot be determined satisfactorily on rabbits with common duct fistulas. But we have already recorded in table 2 that 3 rabbits in the state of experimental alkalosis had gallbladder biles with an average P_{H^+} reading of 7.6. A similar H-ion concentration can possibly be obtained more readily and with less danger in feeding a base-forming diet, like cabbage and carrots. We will have occasion to consider this point repeatedly in other papers of this series. Judging from a few controlled experiments, a state of acidosis develops during starvation which influences very little the reaction of the hepatic duct bile. This corresponds with the observations of

¹⁶ J. Biol. Chem., 1918, 33, p. 215.

¹⁷ Ibid., 1919, 38, p. 539.

Asada.¹³ The cystic bile is, however, always distinctly acid. A reduction of the plasma bicarbonate concentration is in our experience, so far as the rabbit is concerned, followed by a high H-ion concentration of the cystic bile.¹⁸ The hepatic duct bile remains unchanged. The factors which are responsible for the difference can be determined only by further extensive experimentation.

Experimental cholesterolemia has no influence on the reaction of the hepatic duct bile. The low P_{H^+} of the cystic bile of several rabbits is probably the result of fasting or slight under-nutrition. The majority of the animals took the lanolin or brain-carrot mush rather reluctantly, refusing it frequently for several days. The blood of the successfully cholesterolized rabbits contained over 10 times the amount of cholesterol ordinarily found (Bloor¹⁹ — 42 mg. per 100 c c). Hepatic duct bile never contained cholesterol in amounts exceeding 100 mg. per 100 c c of bile.

THE RESULTS WITH GUINEA-PIG BILE

In the course of our experiments we tested 47 gallbladder and 10 hepatic duct bile samples of healthy guinea-pigs kept on a liberal diet of hay, oat and greens. In accordance with the findings of Nichols, we found this secretion to be alkaline to phenolphthalein; the titrable alkalinity varied from 0.43 to 1.8 or 0.154 to 0.720 mg. of HCl per 1 gm. of bile. The P_{H^+} was always above 7.0 and H-ion concentration decreased rapidly in a similar manner, as determined for rabbit bile on standing exposed to air. It was practically impossible to collect the hepatic duct bile without exposure to the air. The 2 successful collections gave readings of P_{H^+} 7.7 and 7.8. When compared with the cystic bile of the same animals, we found that the P_{H^+} of the latter was only 7.2 (normal— P_{H^+} 7.55). These figures suggest, but do not prove, that hepatic duct bile is slightly more alkaline than cystic bile. In the majority of instances, however, the cystic bile differed from the hepatic duct bile neither in its reaction, color nor physical consistency. In the animal body the H-ion concentration is probably identical with that of the blood. Guinea-pigs suffocated or moribund with a blood P_{H^+} of 7.0-7.2 due to an accumulation of carbon dioxide gave gallbladder specimens which were neutral or slightly alkaline (P_{H^+} 7.0-7.1) when tested

¹⁸ Hirsch: Jour. Am. Med. Assn., 1920, 75, p. 1204.

¹⁹ J. Biol. Chem., 1916, 24, p. 227.

immediately. The data available illustrate, however, the progressive decrease on standing.

THE RESULTS WITH DOG, CAT, GOAT, RAT, MONKEY, OX, SHEEP AND PIG BILE

The measurements of the H-ion concentration of the hepatic duct and cystic bile of a series of different animals gave varying results. Two facts were demonstrated: 1. The reaction of the gallbladder bile is always more on the acid side and varies more than the hepatic duct bile. 2. Both secretions change their reaction on standing. The decrease in the concentration is apparently more rapid, and a lower P_{H^+} value, namely 8.2-8.4, is reached in dogs (five) fed and injected intravenously with sodium bicarbonate. Our findings corroborate fully the observations of Okada,¹⁴ who states that it was necessary to keep dog bile free from air, otherwise the value of the H-ion concentration is found to be subject to change.

In connection with our problem on the antiseptic effect of bile, it was of particular interest to record the progressive decrease in the H-ion concentration of gallbladder bile of oxen. Some samples of bile collected aseptically from the gallbladder in from 1 to 2 hours after death and stored in cotton stoppered test tubes, reached a H-ion concentration of P_{H^+} 8.4-9.0. The entire bacteriologic literature dealing with the antiseptic and the inhibitive effect of ox bile has apparently overlooked this phenomenon. Invariably heated sterilized ox bile collected in San Francisco abattoirs has a reaction which certainly cannot be considered ideal for the growth of organisms of the typhoid-paratyphoid group, and yet no attempts have been made to adjust this important factor. Is it not possible that some of the reports and the various contradictory statements relative to the germicidal effect of ox bile can be ascribed to this unrecognized factor? Is perhaps the reduction of the inhibitory effect of ox bile on *B. typhosus* by the addition of glycerol (E. E. Ecker²¹) explained by the fact that impure glycerol is slightly acid and could therefore improve an alkaline bile specimen as a culture medium. All of these questions will be answered in the next paper, but it is sufficiently manifest that attention should be called to the alkaline reaction of old ox bile and to the possibility that chemical transformation takes place more readily in such an environment than when the medium is neutral.

²¹ J. Infect. Dis., 1918, 22, p. 95.

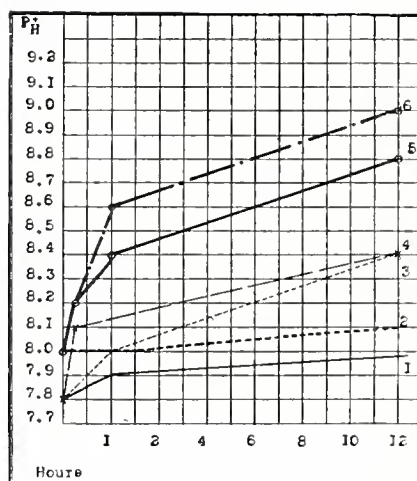


Chart 1.—Change in H-ion concentration of hepatic duct bile on standing at room temperature. 1, rabbit 1481—collected under paraffin oil sealed with rubber stopper; 2, rabbit 1481—corked with rubber stopper, a few bubbles at the top; 3, rabbit 1182—partially filled tube, sealed with rubber stopper; 4 rabbit 1182—partially filled tube corked with cork; 5, rabbit 1200—exposed to air, plugged with cotton; 6, rabbit 1200—exposed to CO_2 free air in desiccator.

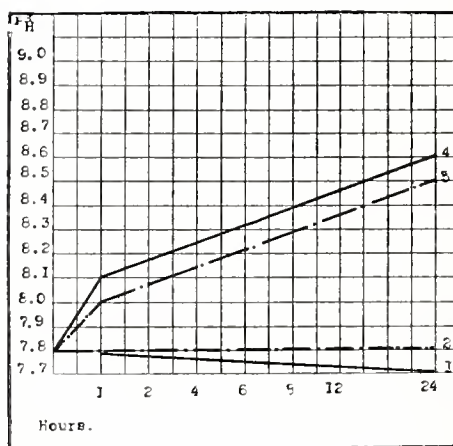


Chart 2.—Change in H-ion concentration of gallbladder "carrier" bile on standing at room temperature. 1, rabbit 1625—bile containing 22,500,000 *B. typhosus* per c.c. kept under sterile condition in the removed gallbladder; 2, rabbit 1630—bile containing 80,000,000 *B. typhosus* per c.c. kept under sterile condition in the removed gallbladder; 3, rabbit 1630—bile in small tube closed with cotton stopper then incubated and found sterile in 4 days; 4, rabbit 1625—bile in small tube closed with cotton stopper, then incubated and found sterile in 15 days after removal.

The only gallbladder bile specimens which remained unchanged on standing or showed after 24 hours an increased H-ion concentration were derived from sheep. In tightly corked tubes the P_{H^+} decreased from 7.2-6.9. This indicates that there is not only no loss of CO_2 , but there also is probably a formation of lactic acid on standing. Further tests will tell whether this increase in the H-ion concentration is characteristic for gallbladder bile of sheep.

Bile samples obtained by fistula from rats behaved like liver bile and were decidedly alkaline. The gallbladder bile of monkeys was acid or alkaline; in one instance the hepatic duct bile was found alkaline.

In connection with our colorimetric measurements of the H-ion concentration of these bile specimens we conducted a series of titration tests. We can in general confirm the observations of Jolles²² Chittenden and Albro²³ and others. The cystic bile of oxen, sheep, goats, pigs, cats, dogs and monkeys is acid to phenolphthalein. From 0.22 to 0.76 mg. of KOH or NaOH were required to neutralize the acid salts of 1 gm. of the different specimens. This acidity varied considerably for the individual animal of the species, and it is apparent that the same factors cited for the differences in the H-ion concentration, namely, the general health of the animal and its diet, influence the titrable acidity or alkalinity. To these two factors we desire to add a third, namely, the time period after death elapsing between the collection of the biliary secretion from the gallbladder gradually becomes more acid. Those exposed to the air in test tubes become more alkaline. The data on which these statements are based was obtained in titrating the specimens as fresh as possible, at least within two hours after death, and immediately after their collection from the gallbladder. Adherence to this principle may in part explain the lower acidity noted in our series in comparison with findings reported by Chittenden and Albro²³ and those of Jolles.²²

The titration method also shows that the hepatic duct bile is different from the gallbladder bile. Invariably the former is very slightly acid to phenolphthalein; the majority of the 1 gm. bile samples required less than 0.1 mg. of NaOH for neutralization. These specimens were uniformly alkaline to litmus.

RESULTS WITH HUMAN BILE

As we found that the reaction of gallbladder bile retained in the rabbit body for some time was subject to change, we considered it

²² Arch. f. d. ges. Physiol., 1894, 67, p. 1.

²³ Am. J. Physiol., 1898, 1, p. 307.

unnecessary to study specimens collected from necropsy examinations. Aside from a few samples obtained from gallbladders after cholecystectomy, we were fortunate in having a case of biliary fistula in the wards. The biliary secretion was collected for a number of days, and the reaction of the same was determined by the titration and by the colorimetric method. The patient felt more comfortable, and the liver bile was less mucoid, when sodium bicarbonate (6 gm. daily), Carlsbad salts and magnesium sulfate were freely administered. This alkaline therapy has to be taken into consideration in judging the reaction of the bile, which was always alkaline to litmus; 1 gm. of bile required on the average 0.72 mg. of HCl to neutralize the mono- or di-basic phosphate salts, using lacmoid as an indicator. As a rule, the clear, light greenish fluid was either neutral to phenolphthalein, or 0.04 to 0.08 mg. of NaOH were necessary to neutralize the specimen using the same indicator. The P_{H^+} was usually 8.0 and increased on standing to 8.6. Heated specimens always had a P_{H^+} above 8.4. The latter procedure was necessary to insure sterility of the biliary fluid, which contained a few paracolon bacilli and *B. coli aerogenes*. The few gallbladder specimens obtained from cholecystectomy cases were alkaline to litmus. The H-ion concentration was low; the range extending from P_{H^+} 7.7-8.6. We have the impression that the specimens were actually liver bile recently discharged into the gallbladder. The bile was light colored, limpid and of low concentration.

In general, our findings agree with those already published. Pisenti,²⁴ Brand,²⁵ Copeman and Winston²⁶ found human fistula bile to be neutral or faintly alkaline; Fränkel and Krause,²⁷ Toida²⁸ and others state that the majority of human gallbladder biles were neutral, a few alkaline, but never acid to litmus.

THE RESULTS WITH GALLBLADDER BILE OF RABBIT TYPHOID OR PARATYPHOID CARRIERS

In examining the gallbladder bile of "carrier" rabbits, we employed the methods outlined in the previous paragraphs. Since 1917 more than 50 specimens have been carefully tested, but only an unselected number of 27 are tabulated in table 4. We found that "carrier biles" differed in no respect from those obtained by fistulas, the P_{H^+} being on the aver-

²⁴ Arch. Med. Ital., 1890, 14, p. 13.

²⁵ Arch. f. Physiol., 1902, 90, p. 491.

²⁶ J. Physiol., 1889, 10, p. 213.

²⁷ Ztschr. f. Hyg. u. Infektionskrankh., 1889, 32, p. 97.

²⁸ Arch. f. klin. Chir., 1913-1914, 103, p. 407.

age 7.5 immediately on withdrawal from the laparotomized chloroformed animals. In not one instance did we find a bile specimen in which the P_{H^+} was below 7.0; in most instances the P_{H^+} was 7.3-7.6 (normal P_{H^+} 7.22). On standing these samples usually decreased in a shorter time than normal hepatic duct bile and reached P_{H^+} 8.0 within 1 to 2 minutes and above 8.6 in less than one hour. Beckwith²⁹ made the same observation. Immediately tested all specimens were alkaline to litmus and were either alkaline (0.1) or neutral or very slightly acid to phenolphthalein. In consistency the biliary secretions were limpid, clear and light greenish. These were at times free from pigment, with a sand-like, yellowish-green granular carbonate sediment (up to $\frac{1}{3}$ of the total fluid bulk) and microscopically had a débris which consisted of leukocytes, epithelial cell and masses of bacteria. The samples rich in carbonate débris, as is to be expected, changed their reaction rapidly to a low H-ion concentration. Others were slimy and pus-like in character and retained their acidity for a longer period than the clear limpid fluids; the H-ion concentration either remained stationary or increased slightly. This phenomenon is shown in chart 2, curve 2. A carrier bile rich in exudate behaves, therefore, in a somewhat similar manner to spinal fluids derived from meningocococic meningitis (Levinson³⁰). It is not unlikely that the mechanism underlying the slow decrease in the H-ion concentration is the same, namely, lactic acid formation due to a destruction of cells on standing. However, owing to the rather small number of purulent carrier biles studied, we are not prepared to offer this suggestion as a final, conclusive statement.

The H-ion concentration of carrier bile stands apparently in close correlation with the physiologic activities of the mucous membrane of the gallbladder. Thickening of the wall with signs of an inflammatory reaction always revealed a limpid bile which behaved similarly to hepatic duct bile. The gallbladder of a "carrier" animal has apparently lost its concentrating function and therefore fails to change the reaction of its content. On recovery from the carrier state, which leads to sterile bile samples and which is accompanied by an increased viscosity and pigmentation, the H-ion exponent returns to the normal variant P_{H^+} range from 6.8 to 7.6. As determined repeatedly, the mere presence of bacteria in the gallbladder bile does not influence the H-ion concentration. A rabbit, for example, in a fasting condition with a typhoid focus in the

²⁹ Thesis, 1920.

³⁰ Cerebrospinal Fluid, 1919, p. 142.

liver, which constantly discharges *B. typhosus* into the bile, but without involvement of the gallbladder wall, may give a P_{H^+} reading of 6.4-6.8 plating of the biliary fluid, may demonstrate millions of viable bacteria. It will be our object in the next paper to consider the relationship of the reaction of the standing carrier biles to the progressive sterilization from *B. typhosus*. Early in our studies we noted that irrespective of the low H-ion concentration, the bacteria present in carrier bile may remain viable for more than 20 days. These observations cast considerable doubt on the conception that the reaction of the bile is the most important factor in the development of a carrier state in rabbits. This drift of thought prompted the diversified studies given above, which in our opinion have shown that in all probability the H-ion concentration of the gallbladder is most suitable for the development of bacteria of the typhoid-paratyphoid group. With the progress of the infection, when changes in the gallbladder wall ensue, the H-ion concentration becomes more or less identical with the one of the blood.

We were unable to correlate the presence of agglutinins in carrier biles with the reaction of the same specimen. Most of our samples were kept in the ice-chest or at room temperature for at least 24 hours before they were used for serologic tests. Their reaction was constantly above P_{H^+} 8.0; low or high agglutination tests were encountered. The H-ion concentration, therefore, cannot be considered the factor which governs the agglutination reactions of carrier biles.

DISCUSSION

The reaction of the bile of laboratory animals is treated only in a few scattered statements of the literature. Unfortunately, these notes are not strictly comparable on account of the variety of methods, which are used for the determination of the reaction. In most instances no distinction is made between the hepatic duct and the cystic bile.

Chittenden and Albro²³ measured the alkalinity of rabbit bile flowing directly from the liver through a fistula and not coming in contact with the gallbladder. They found that it had an alkalinity of 2.5 mg. HCl per gm., using phenolphthalein as an indicator. There was no measurable acidity, and these workers therefore concluded that this bile may contain an alkali as strong as sodium carbonate. Nichols¹ titrated his bile samples using phenolphthalein and lacmoid as indicators and noted a neutral or 3 to 6.0 alkaline reaction in rabbit bile. Aside from Quagliariello,³¹ who reported the H-ion concentration of gall-

³¹ Rendiconti Acc. dei Lincei, 1911, 22, p. 302.

bladder bile of the rabbit to vary from P_{H^+} 6.4 to 7.9, no records of similar determinations by modern methods are available. Apparently no attention has been paid to the changes occurring on standing, and we are therefore unable to establish definitely from the data collected the normal reaction of the liver and of the gallbladder bile of rabbits. Neither could we find statements made with regard to the diet and the general conditions of the animals, which were used for the determinations of the bile reactions.

Our findings as a whole support the contention of Chittenden and Albro.²³ The fistula bile of rabbits is alkaline to phenolphthalein and this alkalinity is probably due to carbonates, as base-forming diets increase noticeably the average titrable alkalinity. The H-ion concentration of the hepatic duct bile varied between P_{H^+} 7.6-7.9; it was never below P_{H^+} 7.5. We are fully convinced that if technical difficulties could be overcome, the reaction would be found to correspond with the one determined for the blood, which according to Hasselbalch³² is P_{H^+} 7.33, (at 38 degrees) and for the rabbit P_{H^+} 7.65 (Dragstedt³³). A well balanced equilibrium apparently exists between the CO_2 tension of the blood and the biliary secretions. This explains our difficulties in changing the H-ion concentration of hepatic duct bile by various diets, HCl feeding or intravenous injection of $NaHCO_3$, even when the titrable acidity or alkalinity differed from the normal average. Misleading values of the H-ion concentration can, however, be obtained when the bile samples are exposed to the air for some time; low concentrations are regularly recorded. We found that the P_{H^+} ranged from 8.2 to >8.6 ; readings of over 8.2 being sometimes obtained 10 minutes after removal from the animal body. Our tests have shown that in the first place these changes follow the escape of CO_2 ; then possibly the absorption of ammonia.

Gallbladder bile of rabbits was found in a large series to be acid to phenolphthalein. Colorimetric determinations, on the other hand, have shown that these biles can be acid, neutral or alkaline. The P_{H^+} range extends from 5.7-7.7. The variability of the reaction exists in individual rabbits and seems to be connected with the general health and the diet of the animals. Fasting, disease and acid-forming diets and HCl feeding produce an acid reaction in contrast to the alkali increasing effect of base-forming diets. Also the cystic bile changes on standing,

³² Biochem. Ztschr., 1911, 30, p. 317.

³³ Jour. Infect. Dis., 1920, 27, p. 459.

perhaps less rapidly and inconstantly than the hepatic duct bile. The final H-ion concentration of such samples does not decrease below P_H^+ 8.0-8.2. This is particularly true when the specimens have been retained in the gallbladder for at least 2 to 6 hours. Concentrated, viscid biles are also less subject to changes than the more dilute limpid specimens. It will be clear from this statement that the cystic bile of rabbits never reaches even in vitro a reaction which is destructive to bacteria, for example, *B. typhosus*. In most instances in vivo the H-ion concentration of the gallbladder bile will be P_H^+ 6.8-7.0. Schoenholz and Meyer have shown that this reaction in salt-free buffered broth favors the optimum growth of *B. typhosus*. In the light of all these findings it is not logical to accept Nichols's conclusions based on in vitro experiments that rabbit bile is antiseptic on account of its alkalinity.

As far as we have been able to survey the literature, no one has as yet determined the H-ion concentration of rabbit carrier bile. We found that such biles behave like the hepatic duct specimens or biologic fluid rich in leukocytes. On standing they either show decreasing, stationary or increasing H-ion concentrations. In vivo the reaction is probably identical with the one characteristic for the blood of the individual rabbit and most suitable for bacterial growth. In vitro a reaction is sometimes reached which must be considered unfavorable for the bacteria of the typhoid-paratyphoid group. Germicidal properties develop only when other factors have made their occurrence, as will be discussed in detail in the next paper.

Nichols first called attention to the strongly alkaline reaction of the guinea-pig bile. As far as titrable alkalinity is concerned, we can fully support his findings; however, the H-ion concentration is high for freshly measured biles and decreases rapidly on standing. In the guinea-pig body the reaction is identical with the one of the blood.

Our H-ion concentration measurements on dogs, cats, goats, oxen, sheep, pigs, rats, monkeys and human bile correspond with those reported by Okada¹⁴ and Quagliariello.³¹ The reaction of the bile from the gallbladder and from the hepatic duct is different. The reaction of the former is more variable and inclines toward the acid side. The general condition and diet of the individual of the same species are in part responsible for this variability. And again, the average titrable acidity or alkalinity varies for different species. With the exception of sheep bile, most of the gallbladder biles exposed to the air show a decrease in the H-ion concentration on standing. The escape of CO_2

is responsible for this change and is particularly striking in ox bile. This fact should be carefully considered in future studies on the inhibitive and antiseptic affect of this secretion.

The hepatic duct biles of dogs, cats and one human case of biliary fistula were slightly alkaline when tested immediately on withdrawal from the body; they also changed on standing. A carefully adjusted equilibrium between the H-ion concentration of the blood and the liver existed apparently also in these species, just as we discussed it for the rabbit and guinea-pig. The liver bile of dogs which are in a state of experimental alkalosis shows a more rapid fall in the H-ion concentration than the hepatic duct bile of the normal.

The numerous experimental bile collections made in the course of our study, supply a number of physiologic data, which have not only a bearing on certain phases of our problem but contribute to the knowledge of the function of the gallbladder and the importance of the biliary secretions in general. These facts are briefly enumerated in the following paragraphs.

RATE OF BILE-FLOW AND THE INFLUENCE OF CHOLAGOGUES

Any one working with animals possessing a biliary fistula is at once struck by the fact that the small herbivorous animals, rabbits and guinea-pigs, secrete considerably more bile in a given time interval than the omnivorous and carnivorous species. When comparing our quantitative findings, recorded in tables 5 and 6, it will be seen that the guinea-pig secretes usually more than twice the amount of bile in 24 hours that the rabbit does. Our figures are based on a considerably larger series of animals than those published by Heidenhain³⁴ in 1883 and by Mann³⁵ in 1918. Their estimates are not strictly comparable with our own for these reasons: Mann made the collections from animals while under an anesthetic, which in his opinion probably decreased the secretion. The data presented by Heidenhain were presumably collected from the work of Bidder and Schmidt³⁶ and were made in a period (report published 1852) when anesthetics were not regularly used in animal experiments. It is our custom to let the animal completely recover from the ether before the rate of bile flow is estimated. We possess, however, sufficient data which show that this operative procedure had but a slight influence on the output of bile per hour. In contrast to Mann, we

³⁴ In Hermann's Handb. d. Physiol., 1883, 5, Pt. 1, pp. 249 and 412.

³⁵ New Orleans Med. & Surg. Jour., 1918, 71, p. 80.

³⁶ 1852, p. 191.

determined the bile secretion for at least 6 hours and estimated our total daily output from this figure. We have gradually become accustomed to consider the average hourly output as 10 c c for rabbits and guinea-pigs. There are individual differences, and temporary reflex inhibition or dislocation of the cannula may influence the total collection. According to our figures, the daily output of bile represents $\frac{1}{8}$ to $\frac{1}{10}$ of the total body weight of the animal (table 7).

The rate of flow is not perfectly uniform. Some animals show a decided decrease in secretion toward the 5th and 6th hour of the experimental period, others a marked increase. These differences are more or less individual, and their cause cannot be easily explained. Improperly secured and restless animals increase by their bodily movements the rate of the bile flow. The diet changes the total 24-hour output. A strict oat diet with a liberal amount of water apparently stimulates bile secretion. The base-forming diet, like hay, cabbage and carrots, produces on the average 80 to 90 c c of bile per 1 kg. weight of rabbit in 24 hours. It is not unlikely that the findings of Bidder and Schmidt ³⁶ are partially explained by the fact that their experimental rabbits were kept on a liberal hay and oat diet. Our observation corroborates the statement made by Abderhalden ³⁷ and suggests that in future determinations of the rate of bile flow proper attention should be paid to the diet of the animals. Unfortunately only two experiments on fasting rabbits are available for comparison. In both rabbits the rate of bile flow was so markedly decreased that even the use of cholagogues failed to increase the hourly output to a noteworthy degree.

As already stated, the reflex inhibition influenced the rate of bile flow in the dog, cat, goat and monkey. Accurate determinations of the amount of bile secreted are therefore impossible.

In the 2 rats successfully operated on and kept under observation for from 12 to 24 hours, the flow of bile was irregular; in one animal the hourly output at the beginning of the experiment was more than 1 c c, in the second it was less than 0.5 c c. The total amount of bile collected in 24 hours represents about one-ninth to one-tenth of the body weight.

In a case of human biliary fistula in which the patient received sodium carbonate and Carlsbad salt, the hourly rate of bile flow fluctuated between 13.2 to 67.4 c c. The total secretion estimated on collections made for 6 hours on 5 successive days averaged 885 c c for 24

³⁷ In Ellenberger and Scheunert: *Lehrbuch d. vergleich. Physiol. d. Haussäugetiere*, 1910, p. 267.

hours. The secretion was never continuous and was clearly correlated with the bodily movements. According to figures published by Cope-man and Winston ³⁸ (779.6 c c for 24 hours), Pfaff and Balch (88) (525 c c) and others our averages appear rather high. It is not unlikely that the alkaline therapy may in part be responsible for this increased rate of bile flow.

The effect on the bile flow of ox bile feeding and the intravenous injection of sodium taurocholate in rabbits was observed in the course of several experiments. The feeding of from $\frac{1}{2}$ to 1 ounce of ox bile in two experiments failed to increase the rate in the 3 to 4 hours subsequent to its administration. Practically the total amount of bile introduced was found in the stomach at necropsy. Intravenous injection of from 200 to 500 mg. of sodium taurocholate produced, as is indicated in table 3, an immediate but temporary stimulus in the rate of bile flow. The hourly average of 5.6 c c increased in one instance to 13.5 c c and in another from 2.8 c c to 9.3 c c. As a rule, the familiar cholagogue action of the taurocholates was spent within the next 2 to 4 hours, depending on the amount injected.

COLOR OF THE BILE

In the course of our experiments we found that the changes in the color of the bile of different species of animals may serve as a valuable guide in judging the age of a specimen. It appears therefore necessary to give a brief description of the various color changes developing a result of oxidative or reductive processes. Rabbit bile secreted from the common duct is clear and has a light green color, which usually deepens to a smaragdine green color on standing. Exposed to the air the sterile fluid acquires in the course of a week a slightly brownish shade. The green gallbladder bile changes more rapidly to a deep brown color. After 2 days usually the bilirubin is transformed to hydrobilirubin. When protected from oxidizing processes by stratification of the sample with paraffin oil, the color remains unaltered for at least 2 weeks.

The hepatic duct bile of the guinea-pig is a light golden yellow color which on exposure to the air becomes a dull green or even a light brown. Cystic bile usually is of the same color; in some animals the secretion may be entirely colorless. A similar color scale can be noted in rat bile.

³⁸ J. Physiol., 1889, 10, p. 213.

The bile of dogs, cats, monkeys and man secreted from the liver is a light brownish yellow, sometimes with a greenish tinge which changes on standing to a dull brown or golden brown. The gallbladder bile of these species, including pigs, may vary from an olive green to a dull reddish brown color, depending entirely on the age of the specimen.

Ox, goat and sheep bile is olive green when fresh, but changes to a dull brown when exposed to the air. Retained in the gallbladder for more than six hours, a similar change is noticeable. The alterations in the color are usually accompanied by definite changes in the reaction of the fluid. This important fact has already been discussed in detail.

THE FUNCTION OF THE GALLBLADDER AND THE DIFFERENCES BETWEEN HEPATIC AND CYSTIC BILE

In the course of a study of the biliary secretions as a medium for the development of bacteria it becomes apparent that certain differences exist between the bile collected from the hepatic duct and the one procured from the gallbladder. Irrespective of the fact that our knowledge concerning the function of the various biliary secretions and particularly of the gallbladder is meager, a brief review of the essential positive findings, as far as they concern our problems, is herewith attempted.

In the light of the work of Mann,³⁵ Rost³⁹ Rous and McMaster⁴⁰ and others it must be recognized that the gallbladder has a mechanical and probably a chemical function. The gallbladder influences the flow of bile and acts as a current regulator of the hepatic duct bile. Observations made by Rost,³⁹ Eisendrath and Dunalvy⁴¹ show that usually all the ducts outside the liver dilate after the removal of the gallbladder. The work of these investigators indicates, that at least in certain species of animals, the gallbladder has a definite function. Okada¹⁴ has demonstrated rhythmic contractions in the gallbladder which increase during the height of digestion and after the administration of acids. Mann³⁵ noted inactivity of the sphincter of Oddi in animals without a gallbladder. The secretions of the bile in such species is continuous. In animals with active sphincters, a gallbladder is necessary to regulate the bile secretion and discharge and to prevent the fluctuations in the intraduct pressure.

³⁵ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1913, 26, p. 710.

⁴⁰ Jour. Exper. Med., 1920, 32, p. 249; Proc. Soc. Exper. Biol. & Med., 1920, 17, p. 159.

⁴¹ Surg., Gynec. & Obst., 1918, 26, p. 110.

The mechanism controlling the action of this viscus is, according to Meltzer,⁴² under nervous control. Disturbances of the law of contrary innervation, in his opinion lead to pathologic stasis. Fasting, starvation or irregular partaking of food does not supply the necessary peptones, which cause the bladder reflex to discharge the bile. In rabbits we have noted the influence of starvation in producing stasis of bile in the gallbladder.

Our observations also confirmed the recent studies of Mann, which showed that the gallbladder of no species of animal is capable of holding more than from 2 to 5% of the total amount of bile secreted in 24 hours. The function of the gallbladder as a reservoir in the same sense as the urinary bladder is not entirely justified, even if we admit that periodical storage in the gallbladder has probably a certain protective advantage to the intestines or some of its functions.

The bile collected from the gallbladder is more concentrated than that withdrawn from the hepatic duct. Hammersten⁴³ found from 1.11 to 1.19% solids in the hepatic and from 8 to 10% in the cystic bile. This difference is the result of the concentrating function and said to be due to an absorption of water, but it is now considered to be caused by the addition of material produced by the cells lining the outer biliary passages. In this way mucous material (mucin, phosphoprotein) and some cholesterol are added to the hepatic bile. Their functional significance is not definitely understood; according to some investigators, the mucus alone is added, and it has no other function than that of anointing the surfaces of the biliary channels and intestines. Rost³⁹ was convinced that the gallbladder bile, which has 10 times more active biliary alkalies and 8 times more solid substances than that of the liver, contains material of greater importance to the digestive tract than the hepatic duct bile. Furthermore, it has been repeatedly noted in rabbits that the contents of a diseased carrier gallbladder are limpid, free from mucus and correspond in every respect with the secretion obtained from the hepatic duct. In such cases the mucous membrane of the gallbladder is practically destroyed by the subacute inflammation provoked by the vegetating *B. typhosus*.

The excellent studies of Rous and his collaborators definitely indicate that the gallbladder mucous membrane is endowed with absorptive properties, which act with great rapidity. Observations on cholesterol-

⁴² *Am. J. Med. Sc.*, 1917, 153, p. 469.

⁴³ *Lehrbuch der physiologischen Chemie*, 1914, p. 390.

ized rabbits by Dewey⁴⁴ and ourselves suggest that cholesterol is deposited on the epithelium of the gallbladder as a result of resorption from the bile and is not a product of secretory activity of these cells. In a few experiments primarily conducted for an entirely different purpose, we found that rabbits eliminated a large percentage of the injected cholesterol through the bile. Particularly in the rabbits, which showed anisotropic fat deposits in the gallbladder, the hepatic duct bile contained a rather high percentage of cholesterol. Experiments on cholesterolized rabbits with common duct fistulas therefore supplied valuable information concerning the mooted origin of the biliary cholesterol.

Various other aspects of the function of the gallbladder, as a reservoir for pathogenic micro-organisms thriving either in its contents or its wall, will be presented in the course of our findings on experimental gallbladder carriers.

SUMMARY

The hepatic duct bile of rabbits is always alkaline to litmus and frequently also the phenolphthalein, the P_{H^+} varies between 7.4 to 7.7, if examined immediately on withdrawal from the body. The H-ion concentration of this bile decreases steadily on exposure to air on standing and may reach a final P_{H^+} of 9.2. If the bile is collected under paraffin oil or put in tightly corked tubes, this change does not take place as readily. The decrease is probably the result of an escape of CO_2 and the absorption of ammonia. The reaction of the bile from the gallbladder is variable; it may be acid, neutral or alkaline, but it is always acid to phenolphthalein. The H-ion concentration of the cystic bile is influenced by the health and the diet of the individual animal and may have a P_{H^+} from 6.4 to 7.7, average P_{H^+} 7.22. On standing also a decrease in the H-ion concentration takes place which is more rapid for animals kept on base-forming diets. Fasting and acid forming diets produce cystic biles of a high H-ion concentration. Feeding of HCl or injection of $NaHCO_3$ may influence the reaction of the gallbladder bile. Alkaline hepatic duct bile, when neutralized with acid and exposed to the air, regains on standing its original low H-ion concentration.

The hepatic duct and gallbladder biles of guinea-pigs differ little in reaction. The bile is strongly alkaline to litmus and moderately so to phenolphthalein. Fresh bile has a P_{H^+} of about 7.5 which changes rapidly, on standing, to a low H-ion concentration.

⁴⁴ Arch. Int. Med., 1916, 17, p. 757.

The reaction of the bile from the hepatic duct and from the gallbladder is different in the dog, cat, goat and monkey. The gallbladder bile reaction is always more variable and inclines toward the acid side. The cystic bile of oxen, sheep, and pigs is faintly alkaline to litmus, the P_{H^+} ranges between 7.0 and 7.5 on fresh specimens but on heating or exposure to the air it changes steadily in some instances to a P_{H^+} above 8.4.

Human fistula bile of one case was faintly alkaline to litmus and faintly acid to phenolphthalein. The P_{H^+} 8.0 increased on standing to P_{H^+} 8.6. Gallbladder bile from cholecystectomy cases showed a P_{H^+} range from 7.7 to 8.6.

Biles derived from typhoid or paratyphoid or streptococcic infected gallbladders of rabbits are alkaline to litmus, and about 50% of them are also alkaline to phenolphthalein. The P_{H^+} varies between 7.3 and 7.6 and frequently decreases rather rapidly on standing. They behave in general like hepatic duct bile specimens. Purulent gallbladder specimens may show, on standing, a stationary or even an increasing H-ion concentration, probably due to the formation of lactic acid provoked by the disintegration of cellular material.

The average hourly rate of the bile flow of rabbits is approximately 10 c c and in 24 hours about $\frac{1}{8}$ to $\frac{1}{10}$ of the body weight. One kg. of rabbit secretes 3.76 gm. and 1 kg. of guinea-pig 6.42 gm. of bile per hour. Intravenous injections of sodium taurocholate produce a temporary cholagogue effect in rabbits, dogs and cats. Particularly in the latter species, the familiar cholagogue effect of sodium taurocholate is of great assistance in overcoming reflex inhibition, which follows operative procedures employed in the production of temporary common duct fistulas.

The mechanical and chemical function of the gallbladder is discussed.

THE BACTERIOSTATIC AND GERMICIDAL PROPERTIES OF BILE

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VII

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SYNOPSIS

Introduction.

Brief review of the literature.

Methods.

The qualitative germicidal action of hepatic duct and gallbladder bile of rabbits, guinea-pigs and dogs.

Notes on the basic qualitative action of hepatic duct and gallbladder bile of the cat, goat, rat, monkey, ox, pig, sheep and man.

The progress of sterilization of naturally infected gallbladder biles of rabbits, monkeys and man in vitro.

Are the antiseptic properties due to bacteriolytic antibodies?

Can agglutinins be demonstrated in bile?

Quantitative tests to determine the influence of reaction on the antiseptic effect of hepatic duct and gallbladder bile.

The rate of growth and the subsequent disinfection in rabbit bile.

The germicidal properties of hepatic duct bile in experimental acidosis and alkalosis.

Progress of sterilization in bile derived from hypercholesterolized rabbits.

Dilution and the addition of serum to hepatic duct bile.

The rate of growth and the subsequent disinfection in guinea-pig bile.

The rate of growth and the subsequent disinfection in dog bile.

The rate of growth and the selective antiseptic action in fresh and old cystic bile of the ox.

The growth of *B. typhosus* and *Vibrio cholerae* in human cystic bile at varying H-ion concentrations.

The problem of the typhoid carrier state, particularly the condition in which the prolonged sojourn of *B. typhosus* occurs in the gallbladder and the biliary passages, cannot be solved conclusively without giving due consideration to the biliary secretions. Some of the factors which are obviously responsible for the prompt localization of the typhoid bacillus, at least in the gallbladder of certain laboratory animals, have been discussed and illustrated in the preceding papers of this series.

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With the exception of Nichols,¹ and quite recently Beckwith,² no investigator interested in the experimental aspect of the carrier state paid sufficient attention to the bile. Nichols claimed that the bile of rabbits and guinea-pigs is strongly alkaline and therefore antiseptic. This conception is rather surprising and contrary to the commonly expressed belief in the literature that bile is a selective stimulating substance favoring the persistence of the typhoid bacillus. However, certain references supply data which confirm the conclusions of Nichols. The following questions, therefore, suggested themselves for investigation: 1. Is the bile of animals and man antiseptic, inhibitive or stimulating? 2. If antiseptic, is the sterilizing effect of bile due to the content of antibodies or due to chemical substances, or both? A study of the phase mentioned under (1) discloses two outstanding facts: First, we are unable to test with the present methods at our disposal the properties of bile *in vivo*. Second, *in vitro* experiments with bile must be considered test-tube artefacts. Our experiments in general support the view that bile *in vivo* is a favorable medium for the typhoid-paratyphoid-group of bacteria.

TABLE 1
BACTERIOLYTIC POWER (?) OF HUMAN BILIARY FISTULA-BILE FOR TYPHOID BACILLI

Case	Treatment	Dosage of Bacilli per C c of Bile	Progress of Sterilization Day					
			1	2	3	5	10	15
L. St. 1917	Daily sodium Bicarbonate Magnesium sulfate and Carlsbader salt	500,000	+	+	+	+	+	+
		50,000	+	+	+	+	+	+
		5,000	+	+	+	+	+	+
		1,000	+	+	+	+	+	+
		100	+	+	+	+	+	+
		50	+	+	+	+	+	+
		25	+	+	+	+	+	+
		10	+	+	+	+	+	+
		5	+	+	+	+	+	+

REVIEW OF THE LITERATURE

Since 1884 nearly 50 authors have tested bacteriologically the properties of gallbladder and common-duct fistula bile of a variety of animals and also of man. The reports can be analyzed only with difficulty, on account of the diversity of methods and bile specimens employed. Some investigators tested fresh bile, others used old heated and sterilized biles in undiluted form, and still others added bile or bile

¹ J. Exper. Med., 1916, 24, p. 497.

² Thesis, 1920.

salts in varying amounts to liquid or solid nutritive substratums. Failure to appreciate the various factors which may alter the same bile specimen in reaction or in chemical composition and lead to a change in its properties as a culture medium, has produced an array of contradictory statements. Quantitative experiments have also been considered in a few instances, and biles collected in different localities have been tested with a variety of pathogenic organisms. Conclusions drawn from observations on ox bile have frequently been applied to other animal biles without the least experimental investigation. Based on the available data, let us see how far we are able to answer the question: Is bile antiseptic or not? Here again we meet with difficulties, because four different properties have been ascribed to the bile *in vitro*, namely, it favors the growth of the common bacteria, except pneumococci and streptococci, or it is indifferent or it can act as an inhibitive or even as a germicidal fluid on the bacterial proliferation. Some experiments have been recorded also, in which the rate of the inhibitive action invariably has led to a complete sterilization of the bile sample. We have observed the four phases in one bile sample and intend to consider the mechanism which produces the various end-results more carefully in the course of the analysis of our data. The findings published deal with the gallbladder bile of oxen, dogs, pigs, man, guinea-pigs and rabbits. Some observations made on fistula bile of rabbits and man are also recorded.

Unquestionably the most detailed studies were made with ox bile. Babes,³ Dünschman,⁴ Fisher,⁵ Lagane,⁶ Neufeld,⁷ Ottolenghi⁸ found it to be a growth-favoring fluid for typhoid, coli and other intestinal organisms, even the cholera vibrio. Bernabei⁹ (not Corrado), Braun,¹⁰ Charrin and Rogers,¹¹ Flu,¹² Gley and Lambling,¹³ Leubuscher,¹⁴ Nichols,¹ Vetrano,¹⁵ Violle¹⁶ report ox bile to be

³ Berl. klin. Wchnschr., 1899, 36, p. 361.

⁴ Ann. de l'Inst. Pasteur, 1909, 23, p. 29.

⁵ Thesis, Bonn, 1894.

⁶ Compt. rend. Soc. de biol., 1912, 73, p. 242.

⁷ Neufeld, O., and Handel, O.: Arb. a. d. k. Gsndhtsamte, 1908, 28, p. 572.

⁸ Centralbl. f. Bakteriol., O. I, 1911, 58, p. 120.

⁹ Ibid., 1892, 12, p. 696; Atti dell Accad. Med. de Roma, 1890-91.

¹⁰ Arch. d. Sc. biol., St. Petersburg, 1901, 8, p. 158.

¹¹ Compt. rend. Soc. de Biol., 1886, 3, p. 425.

¹² Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.

¹³ Rev. Biologique du Nord de la France, 1888, 1, p. 28.

¹⁴ Ztschr. f. klin. Med., 1890, 17, p. 472.

¹⁵ Centralbl. f. Bakteriol. I, O., 1909, 52, p. 275.

¹⁶ Ann. de l'Inst. Pasteur, 1912, 26, p. 381.

indifferent. Copeman and Winston,¹⁷ Ehret and Stolz,¹⁸ Ecker,¹⁹ Fornet²⁰ and Jordan,²¹ Obst,²² Pies,²³ Tonney, Caldwell and Griffen²⁴ proved, however, conclusively, that this secretion is a poor culture medium, and when carefully tested exhibits regularly at least transitory inhibitive properties. Dog bile was found to be indifferent or slightly stimulating by Létienne²⁵ and Miyake,²⁶ while Ehret and Stolz¹⁸ and Toida²⁷ noticed inhibition of bacterial growth. According to Leubuscher,¹⁴ *B. typhosus* and *B. coli* grew well in pig's gallbladder bile.

Fresh human bladder bile can according to Babes,³ Fränkel and Krause,²⁸ Fütterer,²⁹ Hirokawa,³⁰ Kramer,³¹ Leubuscher,¹⁴ Mieczowski,³² Nichols,¹ Pies,²³ Toida²⁷ and others act as a good culture medium. Occasionally samples are found which inhibit strongly the representative organism of the colon-typhoid group (Pies,²³ Hirokawa,³⁰ Toida,²⁷ Copeman and Winston,¹⁷ Ehret and Stolz¹⁸ and Twort and Pastia.³³

Guinea-pig's bile added to gelatine was found to be inhibitive by Copeman and Winston. Fresh alkaline fistulous bile of these animals is, according to Nichols,¹ strongly antiseptic. Vallardi and Bezzola³⁴ state that in guinea-pigs the resistance of the intestines to *B. typhosus* is not due to the pancreatic or biliary secretion.

Lange and Roos,³⁵ Rolly and Liebermeister,³⁶ Talma³⁷ and Nichols³⁸ reported the gallbladder bile of some rabbits as slightly or strongly inhibitive for *B. typhosus*, *B. coli*, etc. Vieillard-Baron,³⁹ Vincent³⁹ and Violle¹⁶ found the antiseptic properties to be feeble or absent. Violle (p. 398), expresses himself on this subject as follows: "Il en est de même avec la bile de lapin ensemencée avec les bacilles typhiques, coli et le vibron cholérique. Bien mieux, ce sera un milieu de predilection pour ces éléments bactériens, qui s'y développeront avec une rapidité beaucoup plus grande que dans toute autre substance nutritive." Nichols¹ tested the bactericidal properties of hepatic duct bile and found it strongly antiseptic for *B. typhosus*, *B. paratyphosus* A, *B. coli* and *B. dysen-*

¹⁷ J. Physiol., 1889, 10, p. 213.

¹⁸ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 7, p. 372.

¹⁹ J. Infect. Dis., 1918, 22, p. 95.

²⁰ Arch. f. Hyg., 1907, 60, p. 134.

²¹ J. Infect. Dis., 1913, 12, p. 326.

²² J. Bacteriol., 1916, 1, p. 73.

²³ Arch. f. Hyg., 1907, 62, p. 107.

²⁴ J. Infect. Dis., 1916, 18, p. 239.

²⁵ Arch. de méd. expér. et d'anat. path., 1891, 1, p. 761.

²⁶ Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 479.

²⁷ Arch. f. klin. Chir., 1913-14, 103, p. 407.

²⁸ Ztschr. f. Hyg. u. Infektionskrankh., 1889, 32, p. 97.

²⁹ München. med. Wchnschr., 1888, 35, p. 315.

³⁰ Centralbl. f. Bakteriolog., O. I, 1909-10, 53, p. 12.

³¹ J. Exper. Med., 1907, 9, p. 319.

³² Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 307.

³³ Compt. rend. Soc. de biol., 1911, 71, p. 13.

³⁴ Abstr. in Off. internat. d'Hyg. Publique, 1918, 10, p. 921.

³⁵ Arb. a. d. Gsndhtsamte, 1915, 50, p. 57.

³⁶ Deutsch. Arch. f. klin. Med., 1905, 83, p. 413.

³⁷ Ztschr. f. klin. Med., 1901, 42, p. 354.

³⁸ Jour. Am. Med. Assn., 1917, 68, p. 958.

³⁹ Vieillard-Barron: Thesis No. 1038, Lyon, 1895. Ann. de l'Inst. Pasteur, 1908, 22, p. 341; Compt. rend. Soc. biol., 1916, 79, p. 580. Vincent, M. H., and Marbé: Compt. rend. Soc. biol., 1917, 80, p. 587. Vincent, M. H., and Fauré-Fremiet, E.: Compt. rend. Soc. biol., 1917, 80, p. 589. Vincent, Marbé and Muratel: Compt. rend. Soc. biol., 1917, 80, p. 675.

teriae. The antiseptic action is, in his opinion, largely due to its alkalinity, which also explains his observation that *Vibrio cholerae*, an alkalophilic micro-organism, grew well in fresh rabbit bile. Aside from the carefully studied selective action of bile and bile salts on pneumococci, numerous reports indicate that one and the same sample may be antiseptic for one group and indifferent to another. Equally important and subject to further investigation is the rather superficially studied time rate of the inhibitive action or the velocity of the sterilizing process in various biles. Ehret and Stolz,¹⁸ Fornet,²⁰ Pies,²³ and Ecker¹⁹ supplied in this connection interesting data, which indicate that a small number of organisms is inhibited to a relatively greater degree than the large inoculum which is ordinarily chosen by the laboratory worker.

From this brief analysis it can be concluded that the results obtained by various workers with bile from the same type of animal and with the same bacterium are more or less at variance, but the majority of writers obtained results which indicate that bile in vitro may be more or less inhibitive, even slightly germicidal. Only Talma,³⁷ Violle³⁶ and Hailer and Ungermann⁴⁰ conducted in vivo experiments on the bactericidal properties of rabbit bile by the direct inoculation of *B. typhosus* into the gallbladder content of these animals. For example, Hailer and Ungermann noted regularly a development of the introduced bacilli in the gallbladder bile, irrespective of the number chosen, and 93% of their animals were found infected up to the 31st day after the inoculation. Less uniform were the results obtained by Talma,³⁷ who also injected typhoid and colon bacilli into the gallbladder of rabbits. The following points in his conclusions are of some interest: Rabbit bile contains a substance which inhibits most of the typhoid and colon bacilli. The bactericidal property is probably the result of chemical action and the antiseptic action of rabbit bile varies in individual animals and at different time periods. If we accept, therefore, the conclusions, which are well supported by the recent careful studies of Jordan⁴¹ and Ecker,¹⁹ that bile is inhibitive even germicidal to a certain degree, we immediately have to answer the question: Is this antiseptic effect the result of (1) antibodies, or (2) chemical substances?

The old Italian and French medical literature deals extensively with the antitoxic properties and the virulence enhancing or depressing effect of the bile in general. The reader will find a complete review of this phase of the problem in a recent summary of Posselt.⁴² On the other hand, definite statements with regard to the presence of specific antibodies in the bile are few and contradictory. Vincent³⁹ and his associates report on the demonstration of complement-fixing antibodies in a small percentage of rabbits immunized against *B. typhosus*, but no statement is made that the bactericidal properties of normal bile must be ascribed to such antibodies.

Most of the investigators conclude that the bacteriostatic and germicidal properties are the result of a chemical or of a physical action. In this connection we have to distinguish between an immediate action of the inhibitive substances on the proliferation of the inoculated bacteria and an indirect effect on the fermentative function of the micro-organisms (Roger⁴³). The chemical properties and constituents of the bile, which are supposed to exert an inhibitive action on microbes are the reaction, the bile acids and their salts, the biliary lecithins and cholesterol, the biliary mucus, the lack of nutritive material, the surface tension and the photodynamic action of bilirubin.

⁴⁰ Arb. a. d. k. Gsndhsamte, 1914, 47, p. 450.

⁴¹ J. Infect. Dis., 1913, 12, p. 326.

⁴² Ergebn. d. allg. Pathologie, 1915, 17, p. 783.

⁴³ Presse méd., 1913, 19, p. 137; Arch. de med. exper. et d'anat. path., 1913, 25, p. 430.

In 1886 Charrin and Rogers¹¹ and in 1917 Nichols¹ suspected the reaction of the bile of oxen, rabbits and guinea-pigs to be largely responsible for their germicidal properties on typhoid, dysentery and paratyphoid bacilli. Their findings are in part supported by the observations of Sellards⁴⁴ made on pneumococci. The influence of dehydrated ox bile or a mixture of purified bile salts on bacteria has been studied with variant results by a number of workers. Sodium taurocholate (1-10% solutions) was found by Dünschman,⁴ Mieczkowski,³² Mosse⁴⁵ and Nicolle and Adil Bey⁴⁶ to be either stimulating or indifferent when added to solid or liquid culture mediums, while Bufalini,⁴⁷ Bunge,⁴⁸ Charrin and Rogers,¹¹ Lindberger,⁴⁹ Limbourg,⁵⁰ Leubuscher,¹⁴ Löhlein,⁵¹ Mac Conkey,⁵² Maly and Emmerich⁵³ and Meyerstein,⁵⁴ report the free acids or their salts to be decidedly antiseptic. Sodium glycocholate was, in the experiments of Dünschman⁴ and of Meyerstein⁵⁴ markedly inhibitive. Basenge⁵⁵ considered the biliary lecithin to be a substance of considerable antiseptic power for *B. typhosus*. His observations that a 1% emulsion kills *B. typhosus* in from 30 to 60 minutes is not supported by the statements of Charrin and Rogers,¹¹ who found even a 10% solution to be innocuous. The opinion advanced by Daniel-Brunet and Rolland,⁶⁶ that 1% solution of cholesterol may check the proliferation of *B. typhosus* has recently been confirmed by Manfredi.⁵⁷ G. Mayer⁵⁸ found the biliary mucin to be inhibitive for diphtheria bacilli, but inactive for *B. paratyphoid B.*, *B. coli* and *B. typhosus*. The lack of nutritive substances, particularly in fistular bile, perhaps the absence of vitamins, as suggested from the observations of Pies,²³ Meyerstein⁵⁴ and others, are factors which deserve a more thorough investigation in the future. Decidedly novel and of considerable importance are, in our opinion, the recent suggestions offered by Larson⁵⁹ and associates, who ascribed the lytic effect of bile on pneumococci to be the result of the well-known low surface tension of this body fluid. The cytolytic action of bilirubin was found to be slight by Charrin and Rogers,¹¹ while Sellards⁴⁴ considered the photodynamic properties of the biliary pigments to be quite marked. Under the conditions prevailing in our experiments the action of these substances was negligible.

It has not been our purpose to investigate all the questions suggested by the review of the literature, but rather to verify some of the published facts bearing on the carrier state and to determine by qualitative and quantitative tests on fresh and old bile samples of a large num-

⁴⁴ Jour. Am. Med. Assn., 1918, 71, p. 1301.

⁴⁵ Ztschr. f. klin. Med., 1889, 36, p. 527; Ztschr. f. Hyg. u. Infektionskrankh., 1900, 34, p. 454.

⁴⁶ Ann. de l'Inst. Pasteur, 1907, 21, p. 20.

⁴⁷ Bufalini, G.: Boll. d. Soc. tra i cult. d. sc. med. in Siena, 1884, 2, p. 142.

⁴⁸ Textbook of Physiol. and Path. Chem., 1902, p. 180.

⁴⁹ Upsala Läkaref. Förh., 1884, 19, p. 467.

⁵⁰ Ztschr. f. Physiol. Chem., 1889, 13, Nos. 1 and 2, p. 196.

⁵¹ Klin. Monatsbl. f. Augsnh., 1908, 46, p. 552.

⁵² J. Hygiene, 1908, 8, p. 322.

⁵³ Sitzungsberichte d. K. Akademie d. Wissenschaften, 1883, 87, p. 3.

⁵⁴ Centralbl. f. Bakteriöl., O., I, 1907, 44, p. 434.

⁵⁵ Deutsch. med. Wchnschr., 1908, 34, p. 139.

⁵⁶ Compt. rend. Soc. de biol., 1911, 171, p. 298.

⁵⁷ Riforma med., 1917, 35, p. 849.

⁵⁸ Centralbl. f. Bakteriöl., 1899, 25, p. 826.

⁵⁹ J. Infect. Dis., 1919, 25, p. 45.

ber of animals the rate of the germicidal action. Experiments have also been conducted to explain the factors responsible for the antiseptic effect, whether biologic or chemical.

METHOD OF STUDY

In the preceding paper of this series, we have described in detail the technic applied in keeping and collecting specimens of bile.

In the qualitative tests, immediately after titration, definite amounts of bile, usually 1 c c, were pipetted into cotton stoppered, serologic vials (7.5 x 2 cm.). For the quantitative tests triplicate sets of 5 c c of hepatic duct bile and 1 to 2 c c of cystic bile were aseptically distributed in pyrex tubes (100 x 12 mm.) with or without paraffin oil. One set of tubes was used for the determination of the changes in the H-ion concentration, a second for the same procedure after inoculation with bacteria and a third for plating.

When we began our tests a few years ago, we inoculated the bile samples with one loopful (2 mg. loop, 22 gauge wire made on a No. 2 Czapslewski standard rod) of a 24-hour peptic digest broth culture averaging 800,000 organisms for *B. typhosus*. In later experiments 1 loopful of a 1:1,000 or a 1:10,000 dilution of the same broth culture, approximating from 1,000 to 100 organisms, was used. The seeded tubes were always incubated at 37 C. The progress of the growth or the degree of the sterilization was followed by transferring at regular intervals of 2 hours 1 loopful of bile on to agar slants. It is self-explanatory that the proper inoculation of the bile samples was controlled by streaking an agar slant immediately on seeding of the tubes. Subcultures were made on peptic digest agar P_{H^+} 7.4.

For the quantitative tests, 5 c c of bile were inoculated with 0.1 c c of a 24-hour broth culture diluted with distilled water, 1:10,000 or 1:100,000. These dilutions on the average approximated 300 to 500 organisms per 0.1 c c. At varying time intervals 0.1 c c amounts of seeded bile were plated either directly or in progressive saline dilutions. Invariably the determinations of the H-ion concentrations were made at the same time. In the quantitative tests the tubes were incubated in an open water bath or in a desiccator over $CaCl_2$ in an incubator.

A modification of the Buxton⁶⁰ method as applied to the determination of the bactericidal properties of serum was also used in some of our quantitative experiments. The procedure was as follows: A 24-hour agar slant culture was washed off with distilled water and standardized to 500,000,000 organisms per c c; 0.1 c c of the dilutions ranging from 1:10 to 1:1,000,000 were transferred into 1 c c lots of bile or serum. After an incubation of 5, 12, 24, etc., hours loop transfers were made on agar slants.

Suitable controls for each experiment, whether noted or not, were treated in exactly the same manner as the biles. The controls were both peptic-digest

⁶⁰ J. M. Res., 1905, 13, pp. 305 and 431.

broth P_{H^+} 7.4, 0.01% and Witte's peptone solution P_{H^+} 7.4, and according to the type of the experiment either distilled water or 0.85% saline solution.

In the quantitative test, salt-free buffered broth, according to Dernby and Avery,⁶¹ with hydrogen-ion concentration corresponding to the initial reaction of the biles, namely P_{H^+} 7.6 and P_{H^+} 8.6, was chosen. Some tubes of this medium were stratified with oil. We failed to note an inhibitive influence of this substance on the growth of the bacteria employed in our experiments.

Representative cultures of *B. typhosus* (K. and C.), *B. paratyphosus* A and B, *B. dysenteriae*, *Staphylococcus* and other cultures, were chosen which grew better in an alkaline than in an acid medium. In order to check the behavior of the bacteria in a general manner we selected the alkalophilic *Vibrio cholerae*.

An attempt was made to adapt the *B. typhosus* to an environment of bile by successive passage through ox bile, but this method was discontinued because no difference in adaptability could be noted. It is generally known that *B. typhosus* inoculated directly into the gallbladder grows luxuriantly. When such infected bile samples were used for the seeding of hepatic duct bile, it gave no evidence of having acquired increased resistance by its previous sojourn in the gallbladder.

In recording the results of growth on the slants the colonies were counted if they did not exceed 100, otherwise they were noted as positive. The plates prepared in the quantitative investigations were counted after 36 to 48 hours' incubation.

Our tests are divided into two groups, the qualitative and the quantitative. Having established the existence of an antiseptic action of certain bile specimens, we investigated the factors responsible for this property, dealing in the first place quantitatively with the question of bacteriolysins and immune bodies, and secondly, with the chemical aspect of the germicidal substances.

THE QUALITATIVE GERMICIDAL ACTION OF HEPATIC DUCT AND GALLBLADDER BILE OF RABBITS, GUINEA-PIGS DOGS, CATS, GOATS, RATS, MONKEY, OX, PIG, SHEEP AND MAN

Irrespective of initial H-ion concentration of the hepatic duct bile specimen derived from 45 rabbits and 10 guinea-pigs, the various strains of *B. typhosus* were unable to remain viable for more than 96 hours, if 1 c c lots were used for the tests 5 to 8 hours after collection. On the other hand, gallbladder bile of the same animals that was seeded in the same manner constantly gave positive subcultures for more than 10 days. Hepatic duct bile of rabbits therefore differs fundamentally with regard to its germicidal properties from true gallbladder biles.

The diet apparently influences in a slight degree the sterilizing effect of hepatic duct bile. Base-forming diets, for example, hay, oats, and carrots or cabbage produce a bile which destroys the ordinary stock typhoid strains (K and C) in from 48 to 196 hours. Another strain, more readily adapted to environmental influences, remained viable for

⁶¹ J. Exper. M., 1918, 28, p. 345.

periods exceeding 72 to 156 hours. Acid-forming diets, oats and bread, prolonged occasionally the viability for several days. Heating of the hepatic bile specimens for from one-half to one hour at 56 C. shortened the period of disinfection. Slight differences in the rate of action were apparent when 2 c c or 4 c c samples were seeded instead of 1 c c specimens. The rate of lethal action was extended for from 2 to 5 days. With the exception of strain K, a small number of typhoid bacilli (100-200 bacteria) was more rapidly destroyed than the usual number of several thousand. This observation is quite in accordance with the findings of Ehret and Stolz,¹⁸ of Fornet²⁰ and of Pies.²³

A selective action of the germicidal substances of rabbit hepatic duct bile on various bacteria was also noted. It is most unfortunate that technical difficulties make it impossible to collect a sufficient amount of pure cystic bile for similar extended tests, but it is evident from the few data collected that the selective action is probably entirely absent or at least not as striking as is the case for hepatic duct bile. Paratyphoid bacilli behave similarly to *B. typhosus*; *B. coli* is somewhat more resistant; while *Vibrio cholerae* and *Staphylococcus aureus* must be looked on as somewhat better adapted species for growth and resistance in fistula bile of rabbits kept on mixed or on acid-forming diets. It should, however, be emphasized that even these organisms cannot lead to an indefinite existence on this type of bile, because subcultures can rarely be found positive after 10 days of incubation. The composition of the bile as a result of diet influences the selective action of *Staphylococcus* and *Vibrio cholerae* to slightly more marked degree, and variant results are more frequently encountered than with *B. typhosus*. The dysentery bacilli are considerably less resistant to the germicidal action; they rarely survive, even if inoculated in large doses. The only exception was the observation made on a bile specimen collected from fasting animals in which the bacilli lived for a period of 36 hours.

Guinea-pig hepatic duct bile exhibits greater disinfecting properties against the same bacteria than rabbit duct bile. The time rate of lethal action is shortened to 36 and 48 hours and even for *Vibrio cholerae* it never exceeds 5 days. *Staphylococcus* occasionally remains viable, even when transferred to this bile in rather small numbers. Otherwise the action seems to follow the same course as in the case of the hepatic duct bile of rabbits. It is usually impossible to obtain from guinea-pigs operated on for a common duct fistula sufficient cystic bile for a bacteriologic study. We therefore tested a large series of gallbladder

biles collected from various necropsy examinations on animals submitted to anaphylaxis tests or those which succumbed to suffocation in a shipment. Most of the samples stood at room temperature for 24 to 48 hours, while the set of specimens collected from suffocated animals was inoculated and tested immediately.

Gallbladder bile specimens which appeared physically normal destroyed typhoid bacilli in 24 to 28 hours, that is to say, in a somewhat shorter time interval than hepatic duct bile. Three specimens which appeared colorless and physically changed acted more slowly in from 48-96 hours. The addition of blood prolonged the time rate of lethal action for over 240 hours. Fresh bile of suffocated guinea-pigs was not bactericidal for *B. typhosus* during the observation period of 400 days, but destroyed *Vibrio cholera* in 216 and 312 days, respectively. Old gallbladder bile of guinea-pigs may be antiseptic for cholera vibrios in a somewhat shorter time interval than hepatic duct bile. A few biles inoculated simultaneously while pipetting the samples into test tubes, and incubated at once showed a viability for *B. typhosus* of from 5 to 6 days.

In conclusion, we can state that our studies with bile specimens of rabbits and guinea-pigs have demonstrated that hepatic duct bile collected from rabbits and guinea-pigs in open tubes is antiseptic for *B. typhosus*, *B. paratyphosus* A, *B. dysenteriae*, *B. coli*, *Vibrio cholerae*, and *Staphylococcus aureus*. Pure, fresh cystic bile of rabbits and of suffocated guinea-pigs either lacks this property entirely or shows it in a less marked degree. The action is selective and apparently influenced by the diet. But we desire to emphasize the fact that the time rate of lethal action has never been less than 48 hours for the *B. typhosus* in hepatic bile of rabbits or 36 hours for that of guinea-pigs. Our observations made on a large series of animals differ therefore materially from those reported by Nichols,¹ who claims that with the exception of *Vibrio cholerae* one loopful of *B. typhosus*, *B. coli*, etc., was regularly killed by duct bile in 24 hours. He failed to state in his first paper whether this germicidal action is only to be attributed to the common duct fistula bile or to the cystic bile or to both. After we had called his attention to our results, he corrected this impression by stating that "the effect (antiseptic) was first noted with bile from a common duct fistula. It is not so definite, especially in the case of the rabbit, with bile from the gall-bladder, which is less alkaline and contains more solids than bile directly from the liver."³⁸ In subsequent paragraphs we shall explain

the factors responsible for the differences between Nichols' and our observations, and discuss in detail his conception that the reaction of the bile is responsible for the germicidal action.

Pure gallbladder bile of rabbits *in vitro* has no antiseptic properties. It may be inhibitive to a slight degree or a poorer medium than broth and may even destroy after prolonged incubation (9 to 20 days) at 37 C. a small number of typhoid bacilli, but it certainly exhibits a different action than does the hepatic duct bile. As the conclusions of Nichols are based on the observations with the latter, his arguments that the bile is an important factor in the development of typhoid carriers are materially deprived of support by our own data, a point which will be discussed from various other angles in the course of this study.

The same differences in the sterilizing properties of cystic and hepatic duct bile as discussed for the rabbit and guinea-pig are also present in the dog. Most of the bacteria remained viable in the gallbladder bile for periods extending over 10 days. On the other hand, 4 of the 7 dogs which had gallbladder biles acting indifferently on bacteria, produced antiseptic fistula biles. Typhoid bacilli inoculated in large numbers rarely survived an incubation period of 120 hours. The colon bacillus and staphylococcus resist an exposure for more than 240 days. Heating of the bile to 56 C. for 30 minutes has no influence on the antiseptic properties. Three other dogs furnished hepatic biles which were bactericidal for dysentery bacilli and in one instance also for a small number of typhoid bacilli. The dogs were all kept on a meat and bread diet and had received inoculations of sodium taurocholate for reasons already explained in the preceding paper of this series. We are quite aware of the fact that the treatment with bile salts may have produced an abnormal hepatic bile, and that we were actually dealing in these experiments with test tube artefacts. This is probably true to a certain extent, but on further analysis these observations offer some explanation for the antiseptic properties of bile in general, namely, sodium taurocholate in an alkaline solution is destructive for *B. typhosus* and other organisms. Proper attention must be given to these dog experiments in interpreting the factors responsible for the bacteriostatic and antiseptic properties of bile. Natural gallbladder bile of dogs has bacteriostatic properties and may be considered a fair medium for the development of *B. typhosus*, *B. coli*, etc. According to Toida²⁷ they may remain viable for from 40 to 70 days.

The differences noted in the germicidal behavior of hepatic duct and cystic bile of rabbits, guinea-pigs and dogs suggested some experiments on other animals. With the same technic we failed to detect in cats, goats, rats and monkeys, any bacteriostatic or antiseptic action of hepatic duct bile. At least qualitatively, the liver bile acts similarly to the gallbladder bile. Fresh unheated gallbladder bile of oxen, pigs and sheep collected in December, retained large numbers of viable typhoid bacilli for more than 10 days. This crude method of testing the properties of a bile sample gave the impression that the bile of these animals in comparison with the hepatic duct bile of rabbits, guinea-pigs and dogs is an indifferent or even a good medium for the growth of *B. typhosus*. Quantitative tests to be described later gave us a better insight into the bacteriologic properties of the bile of these animals and contributed also to the understanding of the factors and the mechanism of the striking lethal action of certain hepatic bile samples.

The few tests conducted with human bile specimens deserve brief consideration. It was not our intention to verify the carefully conducted experiments of Fränkel and Krause,²⁸ Hirokawa,³⁰ Toida²⁷ and others, but we were fortunate in having access to biliary secretions which were collected *intra vitam*, and to a case of biliary fistula, on which we were enabled to study the influence of an alkaline therapy on the germicidal action of bile.

The gallbladder specimens were obtained either from cholecystectomized bladders or by aspiration at laparotomy. The samples were sterile and were primarily used for the determination of the H-ion concentration. Typhoid bacilli grew well in these secretions and remained viable for more than 10 days; in the only sample tested with the *Vibrio cholerae* complete sterilization took place on the eighth day. This observation, which confirms that of Toida and which may have some bearing on the cholera-carrier problem, deserves further and more extended investigation.

The hepatic duct bile for our alkaline therapy experiment was collected from a man, 55 years of age, who developed subsequent to cholecystectomy and kinking of the cystic duct, a postoperative fistula. The bile specimens were on several occasions infected with *B. coli aerogenes*. The patient felt completely comfortable only when taking daily Carlsbader salt; the secretion from the fistula was considerably more mucoid and the jaundice more marked during the few days we excluded this medicament. For a period of several weeks daily doses

of 6 gm. of sodium bicarbonate were administered; to this was added magnesium sulfate or Carlsbader salt. We have already discussed in the preceding paper the fact that only slight changes in the reaction were noticeable and that the bile was nearly always neutral to phenolphthalein or 2.2 alkaline to lacmoid with a P_H^+ of 8.0. This bile never exhibited, when repeatedly tested, any germicidal properties, as is shown in table 1. Even such small numbers as 5 to 10 typhoid bacilli grew freely in this medium and remained viable in large numbers for over 15 days. Human fistula bile collected from an intensively alkalinized patient is not antiseptic in test-tube experiments, and it appears to us most unlikely that this property is present in the body. Our observations on rabbits, to be detailed, negative such an assumption.

Qualitative tests with hepatic duct bile collected in test tubes exposed to air from rabbits, guinea-pigs, dogs, cats, monkeys, goats and man, as well as gallbladder biles of the same animals in addition to that of oxen, sheep and pigs, have demonstrated that with the exception of guinea-pig bile, no true germicidal effect was demonstrable in the first 24 hours after the inoculation. The time rate of lethal action varied between 36 and 240 hours for fistula bile of rabbits and dogs, and for gallbladder biles exceeded many times the longest period noted for the hepatic duct biles. There is a significant selective action on *B. dysenteriae* in comparison to *Vibrio cholerae*, but one is unable to state whether this is similar to the one commonly observed with chemical or biologic bactericides, or whether it is due to an entirely new property. It was noted that in hepatic duct bile of rabbits an appreciable increase of organisms occurred between the 5th and 12th hour after the inoculation of the specimen with a few bacteria, and yet transplants from such tubes at the 24th and 48th hour gave complete sterility. Quantitative tests alone can explain these differences and possibly assist in the understanding of the cycle of events which take place in the bile removed from the body and placed in an open test tube.

THE PROGRESS OF STERILIZATION OF NATURALLY INFECTED GALL-
BLADDER BILE OF RABBITS, GUINEA-PIGS, MONKEYS
AND MAN IN VITRO

In the preceding paper we have called attention to the low H-ion concentration of gallbladder carrier bile of rabbits, and we explained this observation by the fact that the gallbladder wall is seriously damaged and that the infected secretion is mainly hepatic duct bile. A

study of the noninfected hepatic duct bile was therefore essential to a clear understanding of the relationship of bile to the carrier state, a phase of the problem which had been qualitatively dealt with in the preceding paragraphs. For this purpose a large series of gallbladder biles collected from rabbits in various stages of infection was tested qualitatively over periods extending to two months. A few unselected observations are shown in table 2.

Nearly one-half of the rabbit gallbladder biles infected for varying time intervals became sterile in from 4 to 10 days. According to the available notes, these biles were limpid, light green and contained macroscopically no pus or carbonate debris. The number of viable typhoid bacilli determined by plating frequently exceeded one million per c.c. The other half of the carrier biles were all more or less purulent or distinctly viscid, some forming, on standing, a heavy sediment of greenish, sand-like carbonate debris. In these samples *B. typhosus* or *B. paratyphosus* remained viable for more than 30 days, irrespective of the agglutinin content. The physical consistency of the bile, and not the duration of the carrier state, governed the persistence of viable organisms. In view of these results it is evident that removal of the bile from the gallbladder and its exposure to air may have produced antiseptic substances. In all probability these did not, and would not, have developed in the same time interval in the gallbladder in vivo. Progressive decrease in the H-ion concentration in pus-free bile specimens is probably one of the factors responsible for this action. In purulent carrier biles in vitro the greater viability of *B. typhosus* is probably explained by an absence of a decrease in the H-ion concentration as a result of constant CO_2 formation by the pus cells in the sediment, or later the formation of lactic acid as a result of a destruction of cells on standing. It is of interest in this connection to recall that in the course of these tests we became convinced that removal from the animal body is conducive to alteration in the physical and chemical composition of the bile, and that exposure to air produces an unsuitable medium for *B. typhosus*. It also became apparent that it is obviously wrong to draw conclusions relative to the action of rabbit bile on *B. typhosus* when the secretion is exposed to air. The time rate of lethal action was probably a variant factor on account of the different composition and content of bile salts.

The observations made on rabbit bile were confirmed by similar tests with guinea-pig and monkey bile specimens. Dog biles were

TABLE 2

PROGRESS OF STERILIZATION OF NATURALLY INFECTED GALLBLADDER BILES OF RABBITS,
GUINEA-PIGS, MONKEYS AND MAN

Rabbit	Method of Infection and When Killed	Agglutination Reactions		Number of B. Typhosus (Polyvalent) per C c of Bile	Incubated at 37 C. Found Sterile on
		Serum	Bile		
1593	Intravenous, killed 120 hours after injection	40,000	100	50,000 per 8 c c no pus	19th day
1595	Intravenous, killed 10 minutes after injection	1:10++	1:10++	1334 per 0.5 c c no pus	10th day
1609	Carrier, 48 days' duration	1:10,000	<1:10	>5,000,000 per c c pus	21st day
1630	Carrier, 117 days' duration	1:8000	1:10++	>5,000,000 per c c pus	>20th day
1591	Intravenous, 96 hours after injection, killed	1:60,000	1:20+++	1,420,000 per c c no pus	21st day
1715	Intravenous, 48 hours after injection, killed	1:10+++	<1:10	140,000,000 per c c	>20 days
1720	Intravenous, 10 minutes after injection, killed	<1:10	<1:10	30 per c c	>20 days
800	Carrier for 84 days, chloroformed	1:1000	About 50,000 per c c	4 days
805	Carrier for 84 days, chloroformed	1:4000	>1,000,000 per c c	4 days
806	Carrier for 83 days, chloroformed	1:6000	>1,000,000 per c c	4 days
912	Carrier for 64 days, died	1:1000	>1,000,000 per c c	>60 days
926	Paratyphoid B carrier 120 days, died	1:4000	>1,000,000 per c c	>20 days
154	Typhoid carrier for 17 days, died	1:80,000	>1,000,000 per c c	>20 days
972	Typhoid carrier for 10 days, chloroformed	1:400	>1,000,000 per c c	5th day
963	Typhoid carrier for 272 days	1:200	1:10++	12,000,000 per c c	9th day
1028	Typhoid carrier for 29 days	>1:20	27,000 per c c	>20 days
1049	Typhoid infection 19 days; purulent, blood, coagula, etc.	>1:20,000	1:600	14,000,000 per c c	>60 days
1083	Typhoid infection, 7 days; died	1:10,000	1:100	63,000,000 per c c	12th day
1160	Typhoid carrier, 65 days; purulent	1:2000	1:10	11,800,000 per c c	>44 days
1164	Typhoid carrier, 65 days	1:2000	317,000,000 per c c	5th day
1491	Typhoid infection 1 day	1:20,000	5,000,000 per c c	5th day
322	Typhoid infection 7 days	1:100	Guinea-Pigs	>1000 per c c	2d day
326	Typhoid infection 21 days; died	∞ B. typhosus	2d day
S.I. Macacus rhesus	Carrier for 14 days; light green, thin bile	1:8000+++	Monkey 1:10	Innumerable	5th day
Mrs. Bl.	Cholecystectomy following typhoid fever (2/26/18):..	Human	30,000,000 per c c	20th day incubator dried out; room temp. >79 days; in ice chest >79 days
3	Intravenous, 24 hours after infection, killed	<1:10	Dogs <1:10	30 per c c	Exposed to air 21 days; paraffin oil >30 days
6	Intravenous, 4 hours after infection, killed	<1:10	<1:10	2 per c c	Exposed to air >30 days; paraffin oil >30 days

always viscid and behaved like true gallbladder biles. The only human bile examined was purulent, and registered, after exposure to air, only minimum changes in the reaction, which naturally favored the viability of *B. typhosus* therein. The draining gallbladder of the patient was found free of typhoid bacilli 28, 36, and 50 days after the specimen for the test-tube experiments was collected, and yet in vitro the organism continued to flourish. We mention this fact merely as a paradoxical curiosity which again illustrates our belief that test-tube experiments are frequently misleading artefacts.

IS THE ANTISEPTIC EFFECT THE RESULT OF BACTERIOLYTIC ANTIBODIES?

The observations of Buxton,⁶⁰ Bull,⁶² and others recently confirmed by Teague and McWilliams⁶³ have shown that normal rabbit serum is capable of killing large numbers of typhoid and paratyphoid A bacilli. The close relationship between the blood and the secreted bile in the liver shown by the experiments dealing with the passage of micro-organisms from the interlobular veins to the bile capillaries suggested carrying out some tests to determine the presence of bacteriolysins. It was also necessary to settle more satisfactorily the questions dealing with the elimination of agglutinins and antibodies in the bile.

According to Vincent¹⁶ and his pupils, who diligently investigated the presence of complement-fixing antibodies and the so-called "substance anti-sensibilisatrice" in the bile of typhoid immune rabbits, bacteria thrive in the bile of such animals on account of the failure of antibodies to reach the gallbladder or because the antibodies are not persistent on account of the continuous flow and renewal of the secretion. The latter supposition is supported by the observation that the fixing bodies are either absent (in 2 only out of 9 biles examined) or disappear rapidly from the viscus. Forster and Kayser state that bile of immunized rabbits occasionally exhibited bactericidal properties in the peritoneal cavity of guinea-pigs when used in a Pfeiffer bacteriolysin test.

The results of our tests with fresh hepatic duct bile of normal rabbits kept on different diets are given in table 3. We also tested the bile of some immune rabbits and guinea-pigs. With the exception of the latter, which apparently destroyed uniformly 1,000,000 organisms in from 24 to 36 hours, the results were practically identical with those illustrated in the table and are therefore not recorded in detail.

⁶² J. Exper. M., 1915, 22, p. 475.

⁶³ J. Immunol., 1917, 2, p. 93.

Rabbits' hepatic duct bile is capable of destroying any number of living typhoid bacilli in from 5 to 7 days when kept in test tubes exposed to air. Irrespective of the bacteria inoculated, growth was always present with as small a number as 5 organisms, although it rarely extended over the 48th hour.

There is an absence of a graded diminution in the number of viable organisms as ordinarily seen in quantitative biologic tests for serum bacteriolysis; on the contrary, a sudden destruction of a large number of living typhoid bacilli takes place on the 5th day. The serum of the same rabbit is capable of destroying 1,000,000 bacteria in 1 c c of serum in 24 hours. Bacteriolytic antibodies in an immunologic sense are not active in rabbits' hepatic bile, and other factors probably explain the interesting bactericidal properties of this secretion.

The same conclusion can be applied to the bile of other animals, namely, the antiseptic property is not due to bacteriolysins or "a substance sensibilitrice."

CAN AGGLUTININS BE DEMONSTRATED IN BILE?

In this connection we investigated the readily demonstrable immune bodies in the hepatic duct and cystic bile of normal, immune and infected rabbits and guinea-pigs. The findings of agglutinins in the gallbladder biles of immunized rabbits (also guinea-pigs?) reported by Cantani¹¹ are contradicted by the observations of Forster and Kayser,⁶⁴ Venema⁶⁵ and Schöbl on rabbits and by Stäubli,⁶⁶ who studied the subject in guinea-pigs. Stäubli pointed out that bile from cadavers collected many hours after death may contain agglutinins and that the collection of bile from highly immunized animals (guinea-pigs) must be conducted with great care in order to avoid the admixture of traces of blood, which may readily produce positive agglutination reactions in the otherwise agglutinin-free bile. The presence of clumped bacteria in the gallbladder content is, however, commented upon by Cushing,⁶⁷ Richardson,⁶⁸ Doerr⁶⁹ and others.

In our tests we followed the customary technic, using formalinized standardized suspensions both for the bile and serum samples.

⁶⁴ München. med. Wehnschr., 1905, 31, p. 1473; 52, p. 1476.

⁶⁵ Berl. klin. Wehnschr., 1906, 43, p. 99.

⁶⁶ Centralbl. f. Bakteriöl., I, O., 1903, 33, p. 375.

⁶⁷ Bull. Johns Hopkins Hosp., 1898, 9, p. 91.

⁶⁸ J. Boston Soc. of Med. Sc., 1898-99, 3, p. 29.

⁶⁹ Centralbl. f. Bakteriöl., I, O., 1905, 39, p. 624.

Seven normal rabbits had neither in the hepatic duct nor in the gallbladder bile agglutinins above the dilution of 1:10. The cystic bile obtained by laparotomy from 3 immunized rabbits in a series of 13 tested, agglutinated formalinized typhoid bacilli in a dilution of 1:60 or even 1:100. The remaining 10 agglutinated in a dilution of 1:10 or not at all. Bile derived from 10 rabbits which had been chloroformed in various stages of immunization or recovery from infection in 2 animals gave a reaction as high as 1:200 and 1:1000, respectively. Three recovered carriers with marked thickening of the gallbladder wall agglutinated in a dilution of 1:400-600. One immunized animal, which died from an intercurrent infection, was necropsied 8 to 10 hours after death and furnished a cystic bile which clumped typhoid bacilli in a dilution as high as 1:1000. Six normal and 8 immunized guinea-pigs gave hepatic and cystic duct biles which were free from agglutinins irrespective of the serum agglutination titer that they possessed. Our observations confirm those of Foster and Kayser⁶⁴ (p. 1476) and indicate that 25% of the immunized rabbits or recovered carriers may possess agglutinins in the bile. The source of these immune substances cannot be determined with certainty. Some of the rabbits with positive bile specimens had liver injuries and showed the lesions of a chronic cholecystitis or had indications of ascites on account of renal injury caused by a preceding typhoid infection. The degree of the agglutination was entirely independent of the serum agglutinins. The absence of such antibodies in the hepatic duct bile is ample proof, in our opinion, that a true secretion of agglutinins in the same sense as we accept it to be the case for the milk does not take place for the bile, at least in rabbits and guinea-pigs. This fact supports our contention that the antiseptic effect of bile cannot be the result of true, biologically demonstrable antibodies.

A few words on the agglutinins of carrier biles should, however, be added in this connection. An extensive series of gallbladder bile derived from infected animals in various stages of the infection were at our disposal. A total of 31 rabbits was examined; 25 animals were chloroformed, 2 laparotomized and 5 specimens were collected after death. Twenty-five, or 78% of the samples, gave distinct agglutination reactions in dilutions above 1:10, nearly 56% of these biles reacted even in a dilution of 1:100 and above. The strongly positive biles were derived from rabbits, which showed gallbladders in a state of stasis, enormously distended and filled with a pigmentless, slightly purulent

bile. The gallbladder wall was often diphtheritic or in old carriers thickened by the inflammatory process. In others the agglutinin content was due to a slight contamination with blood, and in still others the collection many hours after death must be considered responsible for the presence of agglutinins. The number of viable *B. typhosus* present, not the duration of the carrier stage, apparently influenced the antibody content. The correlation of the findings suggests, that prolonged biliary stasis in the diseased gallbladder due to obstruction of the cystic duct by sandlike carbonate debris or a severe necrotizing, inflammatory process in this viscus are conducive to the presence of agglutinins, and explains the regular finding of clusters of typhoid bacilli in the sediment derived from such biles. How far these antibodies influence the course and duration of the carrier state is difficult to say. As a rule, progressive repair of the inflammatory cholecystitis and hepatitis is shown by a return of the normal color of the bile and is usually accompanied by a marked diminution or even complete disappearance of the agglutinins irrespective of the presence of *B. typhosus*. Gallbladders with impaired function, irrespective of their agglutinin containing bile, more often remain infected for longer periods than the unobstructed ones.

QUANTITATIVE TESTS TO DETERMINE THE FACTORS RESPONSIBLE FOR
THE ANTISEPTIC EFFECT OF HEPATIC DUCT AND GALL-
BLADDER BILE OF RABBITS, GUINEA-PIGS, DOGS,
AND OXEN

From the review of the literature we gained the impression that the reaction, the bile salts, the biliary lecithin and cholesterin play either alone or in combination the rôle of disinfecting substances in bile specimens tested in vitro. The selective action and the variant behavior of the biles of the different animals may in part be the result of the composition of the secretion. This is particularly true with regard to the bile salts. According to Hammarsten⁷⁰ human, rabbit, monkey and ox bile contain mostly, and pig bile entirely, the sodium salt of glycocholic acid. On the other hand, in dog, sheep and goat, bile taurocholic compounds either predominate or are found exclusively in many specimens. The antiseptic properties of the various bile salts in different concentrations (a test-tube experiment) will be treated in a separate paper.

⁷⁰ Lehrbuch der physiologischen Chemie., 1914, p. 390. *Ergebn. d. Physiologic.*, 1905, 4, p. 1.

In this connection attention is called to the recent observation of Davis and Hain⁷¹ and to our own, that rabbits' urine possesses a germicidal action, particularly against the organisms of the colon-typhoid group. The antiseptic properties are inconstant in that all urines do not possess equal action nor are all specimens of urine from the same rabbit of like potency. The action is influenced by diet, but is independent of the H-ion concentration of the urine. The nature of the substances responsible for these germicidal properties is as yet unknown. At the beginning of our studies on the antiseptic action of bile we were favorably inclined to suspect an analogy between biliary and urinary antiseptics, but subsequent tests did not support this view. In most of the experiments the urine specimen of rabbits or guinea-pigs, which gave a strongly antiseptic bile, permitted a free development of *B. typhosus* and allied organisms.

This and other observations stigmatized the reaction as the most important factor in the germicidal action of hepatic duct bile. As early as 1886 Charrin and Rogers⁷² came to a similar conclusion and expressed their view in the following sentence (p. 426): "E \acute{s} t-ce \grave{a} sa reaction que la bile en nature doit ce pouvoir antiseptique moindre que celui de quelques unes de ses \acute{e} lements." Nichols¹ was even more convinced that the alkalinity of the hepatic duct bile of rabbits and guinea-pigs was responsible for its germicidal action because the latter disappeared on neutralization. The importance of the reaction was also recognized by Meyerstein⁵⁴ in his tests with various mixtures of bile salts; a neutral or slightly acid reaction favored the growth of *B. typhosus*, while an alkaline reaction was decidedly inhibitive.

In the early period of the research on immunity, von Fodor,⁷² Emmerich⁷³ and others advanced the theory, based on observations made on rabbits infected with anthrax, that the bactericidal properties of the serum are closely connected with the alkalinity of the blood. The influence of the reaction is undoubtedly only secondary and plays only a subordinate r \acute{o} le. This theory has therefore been entirely disregarded. The preliminary bile studies, which have given the desired information to conduct the quantitative tests reported below, are presented in the preceding papers. Having established the optimum H-ion concentration for the growth of *B. typhosus* and also its generation time, we studied

⁷¹ J. Urolog., 1918, 2, p. 309.

⁷² Arch. Hyg., 1886, 4, p. 129.

⁷³ Emmerich, E., and Wagner, G.: Centralbl. f. allg. u. path. Anat., 1916, 27, p. 433. Ztschr. f. Immunitätsforsch. u. exper. Therap., 1916, 24, p. 557.

in detail the changes which take place in the hepatic duct bile of rabbits and guinea-pigs on standing exposed to air or alkali. It was found that on account of the escape of CO_2 , and possibly the absorption of ammonia, the H-ion concentration decreased progressively in 24 hours to a P_{H^+} of from 8.6 to >9.0 ; in a similar manner the titrable alkalinity increased to a marked degree. On the other hand, it was demonstrated that these changes could be retarded by the stratification of the biliary effluent with paraffin oil during the act of collection and during the experiments. We have stated in this connection that the reaction of hepatic duct bile in vitro exposed to air for even a short time differs materially from that present in the animal body. Similar changes in the reaction to a less marked degree occurred in the cystic bile and a final P_{H^+} over 8.4 was occasionally recorded. It was also pointed out that the diet and the biliary stasis provoked by starvation affect the H-ion concentration of the gallbladder bile.

These principles were applied to the study of the antiseptic effect of hepatic duct bile of rabbits and later extended to that of guinea-pigs, oxen and man. Definite amounts (usually 5 c c, cystic bile 1-2 c c) of fresh bile collected under oil, together with broth controls of a definite P_{H^+} , were seeded with 300-500 typhoid or dysentery bacilli or *Vibrio cholerae* and kept in a water bath at 37 C. At varying time intervals 0.1 c c amounts were plated either directly or in progressive dilutions in peptic digest agar. After 24-36 hours' incubation at 37 C. the plates were counted. It is customary to use the logarithm of the number of viable organisms in recording results of experiments on the rate of growth of bacteria. In the accompanying charts the logarithms of the viable organisms per 0.1 c c are plotted as ordinates against time intervals as abscissae. Bile samples, which show by plating active, vigorous growth, are never turbid; they may when exposed to air change their color to an olive-brown, but when stratified with oil they always remained smaragdine green. It was also noted that certain types of bacteria reduced the biliary pigment more readily than others.

From an extensive series of identical tests we select a few illustrative examples.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION OF RABBIT BILE

Chart 1 illustrates the rate of growth of *B. typhosus* in the hepatic bile of a rabbit kept on an oat diet, compared with the one in buffered

salt free broth. Similar experiments conducted with hepatic duct bile of rabbits kept on a mixed and on a cabbage diet gave identical results. Hepatic bile of rabbits, when tested in vitro exposed to air and alkali favors in the first 24 hours the growth of *B. typhosus*; later it acquires germicidal properties, which lead, irrespective of the diet of animals, to complete disinfection of this body fluid in 96 hours. This definite cycle of events, so well illustrated graphically, can be prevented by stratification of the hepatic duct bile with oil. Cessation of growth occurs in the stratified bile at the same time as in the broth, and the disappearance of viable typhoid bacilli progresses in a similar manner. As the typhoid bacilli inoculated in the bile specimens were grown in a broth of a

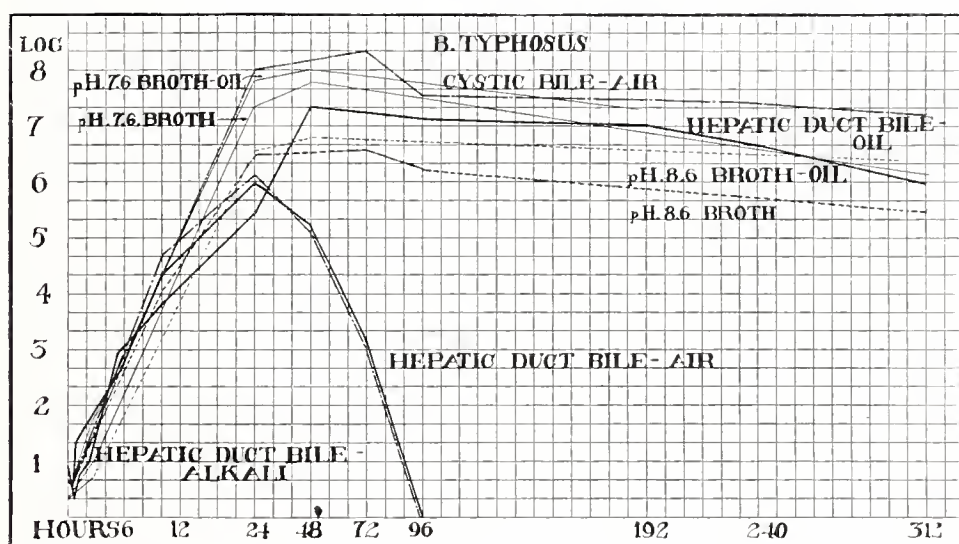


Chart 1.—Rate of growth of *B. typhosus* in the hepatic duct bile of a rabbit kept on an oat diet compared with growth of the bacillus in buffered salt-free broth.

reaction of P_H^+ 7.0, transplantations to mediums of more alkaline reactions (P_H^+ 8.4-8.6) were expected to produce a decided "lag."

If we assume for the present that the nutritive requirements are satisfactory for the growth of *B. typhosus* and recall that this organism has a distinct adaptability to changes in H-ion concentration; although it is more alkalo—than acidophilic, it appears not at all surprising that the initial growth in all the bile specimens is good. Once accustomed to the environment, the organisms continue to grow slightly or remain viable even when the reaction has undoubtedly reached an unfavorable point, above P_H^+ 8.8. The acquired alkali tolerance of *B. typhosus* in milk illustrates this well. Test-tube experiments with a salt-free broth

containing 1% of peptone with phosphate mixtures and sodium hydroxide have shown that the "alkali death point" for *B. typhosus* (K) in such a medium varies between P_{H^+} 9.2 and 9.4. A substratum containing more peptones or colloids pushes the "death point" to P_{H^+} 9.6 or even 9.8.

When bile is exposed to alkali, progressive decrease in the H-ion concentration following the escape of CO_2 may reach its lowest level in from 10 to 12 hours at 37 C.; yet the typhoid bacillus continues to grow only to be inhibited and destroyed 36 to 48 hours later. Other organisms may develop in the same environment for periods extending over 10 days. This phenomenon was called to our attention by the

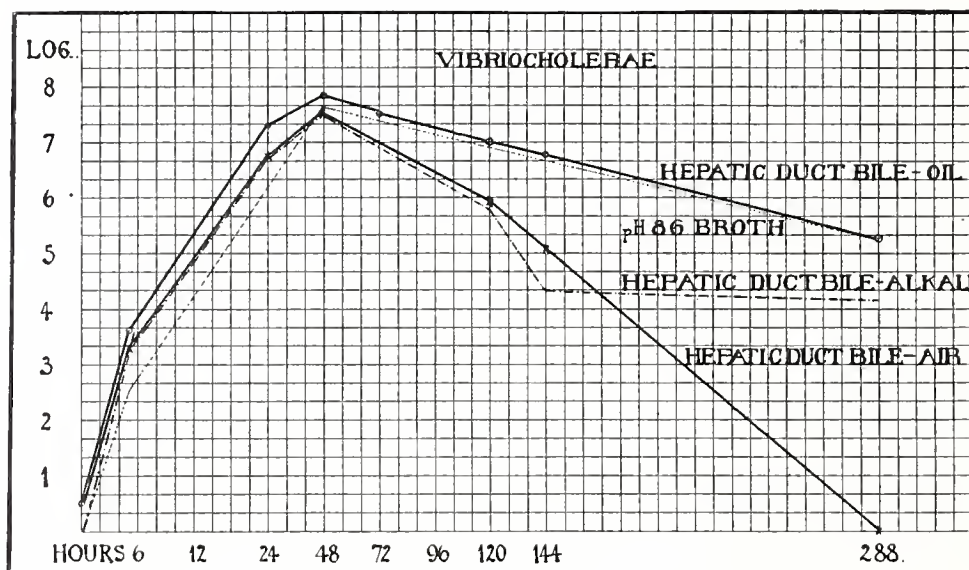


Chart 2.—Rate of growth of *Vibrio cholerae* in the hepatic duct of a rabbit.

experiment illustrated in chart 2. The alkalophilic *Vibrio cholerae* grows favorably in rabbit bile covered by oil and is completely killed in the secretion exposed to air after an incubation time of 12 days. Cessation of growth takes place in all tubes inclusive of the broth control after 48 hours' incubation. The progressive disappearance of viable organisms proceeds slowly in the broth and bile tube stratified with oil, and more rapidly in the tubes exposed to air or alkali. The decrease in the tube kept in the desiccator is apparently interrupted after the 144th hour. In this experiment the tube remained unopened in the desiccator for 6 days and was found to contain a large number of viable vibrios on the 12th day of incubation. The reaction of this specimen

differed only slightly from that exposed to air, and yet the spirillae remained viable. The absence of germicidal properties for *Vibrio cholerae* in bile kept in a sealed desiccator is rather difficult to explain. The nature of the factors operative on the bile in this environment, in contrast to that exposed to air, is not clearly understood. Three possibilities suggest themselves: (1) absorption of water and concentration of the bile (accurate quantitative tests were not made but casual observations failed to support this particular contention); (2) continuous absorption of the ammonia produced by the growth of the organisms; and (3) the prevention of ammonia absorption from the air.

Old bile exposed to the air at room temperature or in the ice-chest is antiseptic the first 24 hours for *B. typhosus* and *B. dysenteriae* Shiga. This observation explains the results of Nichols and our own in the qualitative series. Rabbit hepatic duct bile collected in open tubes and kept at room temperature even for 24 hours will exhibit strongly bactericidal properties; the inoculated bacteria are unable to grow and are progressively destroyed after 15 hours of incubation. Such biles can be partially rejuvenated by saturation with CO_2 and subsequent stratification with oil. Various bacteria are enabled to grow at least for 15 hours but subsequently germicidal substances make their appearance and sterilize the hepatic bile in 48 hours when exposed to air, or in from 192 to 216 hours when stratified with oil.

A similar cycle of events takes place in bile samples which are neutralized by the addition of HCl or lactic acid. These experiments were merely undertaken to verify the observations of Nichols.¹ It is quite obvious that the addition of strong acids, such as hydrochloric and sulphuric as recommended by this worker, may, aside from neutralizing the carbonates, materially change the chemical composition of the secretion and produce conditions which never occur in the animal body. Neutralization of fresh and old bile, which is germicidal in 24 to 96 hours, produces a hepatic duct bile suitable for the growth of a variety of bacteria. Invariably the antiseptic properties manifest themselves in the neutralized biles in from 1 to 8 days later than in the untreated ones. Bile specimens which are neutral show a decline in the H-ion concentration probably due to an escape of CO_2 or to an absorption of ammonia. The inoculated types of bacteria, however, grow well and remain viable for over 10 days. Such biles are antiseptic for *Vibrio*

cholerae. The change in the H-ion concentration is therefore in all probability only one of the many factors which is altered under the influence of the neutralization process.

THE GERMICIDAL PROPERTIES OF HEPATIC DUCT BILE IN EXPERIMENTAL
ALKALOSIS AND ACIDOSIS

In the preceding paper we discussed the experiments which were undertaken with the intention of determining the H-ion concentration of hepatic duct bile under the influence of an experimentally produced alkalosis or acidosis. It was stated that the reaction of this secretion is rarely changed on account of the feeding or the injection of NaHCO_3 or HCl . It was, however, shown that the reaction of the cystic bile of rabbits may be distinctly influenced by the same treatment with these chemicals or by fasting. In the discussion of the qualitative tests reported in the preceding paragraphs of this paper, it was shown that acid-forming diets may produce a hepatic duct bile which becomes only germicidal after 96 to 168 hours' standing at room temperature. These facts naturally suggest some experiments in order to prove or to refute the contention of Nichols that an alkaline therapy may be of value in preventing and curing typhoid gallbladder carriers. Animals kept on acid-forming or base-forming diet were in the course of the experiment treated with alkalies, and the hepatic duct bile collected before and after the administration of the chemicals was submitted to qualitative tests.

The intravenous injection of sodium bicarbonate produced in one rabbit a hepatic duct bile in which *B. typhosus* was killed. From another rabbit a similarly active bile specimen was collected after the administration of 2 gm. of NaCO_3 intravenously and of 5 gm. by stomach tube. An animal kept on a base-forming diet for 16 days and intensively alkalinized before the operation furnished a hepatic duct bile and a cystic bile which differed in no respect from those obtained from rabbits kept on a base-forming diet alone. These few experiments are suggestive, but further tests on a large series of animals are necessary before final conclusions can be reached. It is quite obvious from the observations already reported that we are dealing in those germicidal tests with test-tube experiments, and that we have no knowledge of the physiologic or pharmacologic action of alkalies on the rabbit organs and tissues or on the composition of the secreted bile. Observations to be reported in a subsequent paper indicate that alkalinization of typhoid infected rabbits may act indirectly in preventing the accompanying acidosis and not

through their immediate action on the organisms themselves. It is in this sense that we feel the experiments of Nichols may finally be interpreted, when all the factors concerned have been fully analyzed.

TABLE 3
BACTERIOLYTIC POWER (?) OF HEPATIC DUCT BILE OF RABBIT FOR TYPHOID BACILLI

Rabbit	Diet	Dosage of Bacilli per C c of Bile	Progress of Sterilization Days							Remarks
			1	2	3	4	5	6	7	
1418 Serum agglut: 1:5 Bile agglut: <1:5	Mixed diet	710,000	+	+	+	+	0	Open in tube with cotton stopper One c c of serum destroyed 1,000,000 in 24 hours
		300,000	+	+	+	+	0	
		20,000	+	+	+	+	0	
		1,500	+	+	+	+	0			
		184	+	+	+	0				
		23	+	+	0					
		12	+	+	0					
		6	+	+	0					
		3	+	0						
1418 Serum agglut: 1:5 Bile agglut: <1:5 1181	Mixed diet	160	+	+	+	+	+	+	+	Under paraffin oil
		23	+	+	+	+	+	+	+	
		12	+	+	+	+	+	+	+	
		2	+	+	+	+	+	+	+	
	Oats	500,000	+	+	+	+	0	Open tube; cotton stopper
		250,000	+	+	+	+	0			
		25,000	+	+	+	+	0			
		2,500	+	+	+	+	0			
		500	+	+	+	+	0			
		50	+	+	+	+	0			
		25	+	+	+	+	0			
		12	+	+	+	+	0			
		5	+	0						
1182	Cabbage	847,000	+	+	+	+	+	0	..	Open tube, cotton stopper
		423,500	+	+	+	+	+	0		
		42,350	+	+	+	+	+	0		
		4,235	+	+	+	+	+	0		
		847	+	+	0					
		84	+	0						
1200	Cabbage and NaHCO ₃	500,000	+	+	+	+	+	0	..	Open tube, cotton stopper
		250,000	+	+	+	+	+	0		
		25,000	+	+	+	+	+	0		
		2,500	+	+	+	+	+	0		
		500	+	+	+	+	+	0		
		50	+	+	+	+	+	0		
		25	+	+	+	+	+	0		
		12	+	+	+	+	0			
		5	+	+	0					

As the germicidal action of hepatic duct bile is probably closely connected with the available bases in this secretion, an investigation of the behavior of this fluid obtained from an animal impoverished of these substances was important. A state of acidosis is readily produced in rabbits by feeding hydrochloric acid. The experiments were similar to those reported in the foregoing. The acid was fed to the rabbits operated on after the collection of the necessary control samples.

With the exception of rabbit 1031 b, no appreciable influence on the germicidal properties of hepatic duct bile of rabbits in a state of experi-

mental acidosis could be noted. From this animal, after a total feeding of 0.72 gm. of HCl, a bile sample was obtained which sterilized itself of 2 strains of *B. typhosus* after 192 instead of 144 hours. The viability of *B. dysenteriae* was prolonged for an additional 24 hours. The minor differences recorded for the bile specimens of rabbits 32 and 1118 may be safely attributed to the method of testing the progress of sterilization and not to any material change in the bile itself. It is possible that more definite results would have been obtained if the animals had been kept on acid-forming diets and if the treatment with HCl could have been extended over a longer period. For practical purposes a continuous mild acidosis can probably be more satisfactorily obtained by fasting or by a predominant oat diet.

TABLE 4
THE PROGRESS OF STERILIZATION OF HEPATIC DUCT BILE OF RABBITS DILUTED WITH
DISTILLED WATER
Four Rabbits. Diet: Mixed oats and carrots.

Changes Approximately Produced in Bile	PH	Fistula Bile: 1:1000 to 1:10,000 Loopful of a 24-Hour Broth Culture								
		Ty- phoid K	Ty- phoid C	Ty- phoid J	Ty- phoid B	Para- ty- phoid A	B. Dysen- teriae Shiga	Para- dysen- teriae Strain	B. coli	V. chol- erae
1 c c bile.....	8.4	72	84	96	72	96	24	24	132	228
1 part bile + 1 part dis- tilled water.....	..	150	192	192	192	216	24	24	288	408
1 part bile + 2 parts dis- tilled water.....	..	264	276	204	360	336	48	60	384	408
1 part bile + 3 parts dis- tilled water.....	..	336	288	276	324	360	48	96	384	408
1 part bile + 9 parts dis- tilled water.....	..	264	312	312	408	384	48	156	408	408
1 c c distilled water.....	..	24	24	24	24	24	24	36	132	72
1 c c peptone (0.1%) solu- tion.....	..	400	408	408	408	408	108	240	408	408

DILUTION AND THE ADDITION OF SERUM TO HEPATIC DUCT BILE OF RABBITS

Observations reported above suggest that the germicidal properties of hepatic duct bile in all probability depend on other factors than merely progressively increasing alkalinity. Among the factors to be considered are the lack of nutritive material and the influence of H-ion concentration on the action of the bile salts. The first problem was investigated by progressively diluting bile samples or by adding heated sterile rabbit serum to strongly bactericidal bile specimens and inoculating each specimen with small amounts of typhoid bacilli.

In table 4 we summarized our results obtained on 4 hepatic duct bile samples collected from animals kept on various diets. It is quite evident that the progress of sterilization is retarded for from 3 to 5 days, when the bile specimen is diluted with distilled water in the proportion of 1:1. Moreover, progressive dilution produces a pronounced retardation in the antiseptic effect of one and the same bile sample. The distilled water used was doubly distilled and free from CO_2 . *B. typhosus* and most of the other bacteria refuse to grow in it. These observations clearly demonstrate that the antiseptic properties of hepatic duct bile of the rabbit cannot be the result of a lack of nutritive substances. The H-ion concentration of the secretion exposed to air is only indirectly responsible for the gradual destruction of the inoculated micro-organisms. Even in a poorly buffered medium, such as the bile diluted 1:1 or even 1:2, the recordable change in the H-ion concentration is negligible and for practical purposes has no influence on the viability and growth of the inoculated bacteria. The actual hydrogen-ion concentration is decreased in a dilution of 1:9, and yet the various typhoid strains remained viable for more than 10 days in comparison to the short period of 4 days noted in the control tubes. It is our belief that dilution reduces the main germicidal substances, probably the effect of the bile salts. The time rate of lethal action is prolonged, irrespective of the low, unfavorable H-ion concentration. It is possible that the procedure of diluting bile induces other alterations, which we have not as yet investigated carefully. Even a profound change in the protein and salt content appears to be more favorable for the persistence of viable *B. typhosus* than the concentrated secretion supposedly rich in nutritive material.

In this connection it was naturally of interest to see how far the addition of nutritive material, for example serum, influences the rate of lethal action. According to Pies²³ and others, bile becomes regularly a good culture medium by the addition of organic matter, such as blood serum, pus or aleuronate. In table 5 the average time rate of lethal action of 8 hepatic duct biles diluted with serum on 2 strains of *B. typhosus* and 1 paratyphoid strain is compared with that of undiluted and diluted bile. Contrary to the general belief expressed in the literature, fistula bile fortified by the addition of serum is not a medium in which *B. typhosus* can develop indefinitely. For example, the destruction in a specimen diluted with the serum in the proportion of 1:1 or 1:2 is identical with the one obtained for the same specimen diluted with

distilled water. The prolongation of the viability of the organisms is therefore purely the result of a dilution of the antiseptic substances and not due to the addition of nutritive material or "vitamines." This conclusion is supported by the figures given in table 5; as has already been shown, the addition of small amounts of serum had no influence in its prolific effect. Our data support the conclusion of Pies that dilution with saline solution does not exert the same influence as distilled water. Further experiments are necessary before we feel justified in offering an explanation for this interesting observation.

The next factor or group of factors to be analyzed would be the bile acids and their disinfecting properties at various H-ion concentrations. Certain observations made on dog bile containing an excess of bile salts were so suggestive that we considered it advisable to conduct an extensive series of quantitative tests which will be reported in a separate paper.

TABLE 5
THE PROGRESS OF STERILIZATION OF HEPATIC BILE DILUTED WITH HEATED RABBIT SERUM

Rabbit	Diet	Amount of Heated Serum Added to Fresh Bile	Pa ⁺	Fistula Bile		
				Typhoid Kl 172	Typhoid K 120	Para- dysentery 310
8 Rabbits	Mixed Oats Cabbage and Carrots	1 c c bile.....	8.1	124	110	36
		1 c c serum.....	..	>240	>240	..
		1 c c bile + 1 c c serum.....	..	216	192	..
		1 c c bile + 0.5 c c serum.....	..	192	168	96
		1 c c bile + 0.25 c c serum.....	..	204	168	..
		1 c c bile + 0.2 c c serum.....	..	114	..	36
		1 c c bile + 0.1 c c serum.....	..	104	90	12
		1 c c bile + 0.05 c c serum.....	..	68	84	..
		0.95 c c bile + 0.05 c c serum.....	..	168
		0.75 c c bile + 0.25 c c serum.....	..	192	216	..
		0.5 c c bile + 0.5 c c serum.....	..	266	266	..
		1 c c bile + 0.5 c c saline.....	..	96	96	..
		1 c c bile + 0.5 c c distilled water..	..	168	168	..
		0.5 c c bile + 0.5 c c distilled water	..	216	216	..

THE PROGRESSIVE STERILIZATION IN BILE DERIVED FROM HYPER-
CHOLESTEROLIZED RABBITS

Among the chemical components of the bile the various lipoids, soaps and cholesterol fractions deserve some consideration in the light of recent observations. Manfredi,⁷⁴ for example, reports that a concentration of cholesterol of 0.5% added to culture mediums will check typhoid and the paratyphoids, while *B. coli* will grow in concentrations up to 1%. It is worth noting that Baemeister⁷⁴ and Exner and Heyrovsky⁷⁵ observed the decomposing effect of bacteria of the typhoid-

⁷⁴ München. med. Wchnschr., 1908, pp. 211, 283 and 339.

⁷⁵ Arch. f. klin. Chir., 1908, 86, p. 609.

paratyphoid group on bile, which frequently resulted in the precipitation of cholesterin. The experiments of Bassenge⁵⁵ with lecithins also deserve consideration. This worker demonstrated that these substances in 1% emulsions are destructive for typhoid bacilli in from 30 to 60 minutes. On the other hand, the growth-stimulating properties of unheated lecithin-containing mediums for *B. anthracis* and the tubercle bacillus is well known. Moreover, we recall the studies of Morato and Villanueva,⁴⁵ who demonstrated that cholesterol injections hasten the production of antibodies and agglutinins.

In order to determine the germicidal properties of lipoids and cholesterol we conducted a small series of tests with hepatic duct bile derived from hypercholesterolized rabbits. A fairly marked hypercholesterolemia was readily produced in these animals by feeding either lanolin (Merck's C. P.) or sheep brain or egg yolks. In the preceding paper data were recorded which demonstrated conclusively that it was possible to increase in rabbits the demonstrable blood cholesterol. Such a condition frequently led to a distinct precipitation of this substance in the gallbladder bile. The hepatic duct bile reached on one occasion only a concentration of 0.1% cholesterol, which according to the findings of Manfredi⁵⁷ is insufficient to exert a checking influence on *B. typhosus*. On the other hand, the gallbladder bile gave a concentration of 0.5%, despite which the growth and persistence of the viable *B. typhosus* were unhampered. The biles of cholesterolized rabbits were not any more germicidal than those of normal rabbits kept on various diets. Tests not detailed in this paper have confirmed in a general way these observations, and have shown that biles derived from fasting animals, which invariably have an increased cholesterol content (Rothschild⁷⁶), differ little in their germicidal properties from those of normal animals.

Next in order for investigation would be the biliary mucin and the surface tension. A study of the latter is contemplated. An investigation of the antiseptic properties of biliary mucin appeared superfluous. Hepatic duct bile, which becomes antiseptic, is free from this substance, and cystic bile, which occasionally contains a considerable amount, is a good medium. This justifies the impression that the admixture of this secretion to hepatic duct bile of rabbits is in part responsible for the absence of germicidal properties.

⁷⁶ Beitr. z. path. Anat. u. z. allg. Path., 1915, 66, p. 227.

A number of experiments were made by using ether or chloroform extracts of hepatic duct bile of rabbits. No difference in the influence on the growth of *B. typhosus* was observed in such biles in contrast to those untreated.

THE GROWTH OF *B. TYPHOSUS* IN HEPATIC DUCT BILE ENCLOSED IN COLLODIUM SACS IN THE PERITONEAL CAVITY OF THE RABBIT

It has been shown that hepatic duct bile of rabbit removed from the body becomes antiseptic when exposed to air. In an effort to rejuvenate the old antiseptic bile we returned it to the animal body in collodium sacs. This transformation of germicidal bile into a good medium was apparently accomplished in the following experiment.

Exper. 1.—A paralodium sac prepared over a glass guide with 4 holes sterilized in live steam for 1 hour was filled with 4 c.c. of hepatic duct bile (reaction P_{H^+} 8.4) of rabbit 1182. This bile when tested immediately and when exposed to air destroyed 100 organisms per c.c. in 48 to 72 hours. The bile in the paralodium sac was seeded with an average of 50 typhoid bacilli (Strain K). The sac was carefully sealed and transferred to the peritoneal cavity of rabbit 1190. After 6 days had elapsed this animal was killed and carefully necropsied and cultures made. The tissues were found free from typhoid bacilli. The paralodium sac was covered by a thin layer of fibrin: there was no leakage of the membrane. The contents were pale greenish with a slightly brownish tinge and were perfectly clear. One c.c. contained 2.5 million viable *B. typhosus*. The reaction was P_{H^+} 6.7.

It is evident that old, antiseptic hepatic duct bile in a paralodium sac, seeded with a small number of *B. typhosus* becomes a good culture medium for these organisms when placed in the peritoneal cavity of a rabbit. The most obvious change noted in this bile was an increase in the H-ion concentration; practically the optimum for *B. typhosus* had been attained. Naturally, this is only one of the changes which probably took place, and it would be erroneous to conclude that we reproduced in this experiment the conditions as they exist in the gallbladder. The observation is, however, of considerable interest, and further experimentation, particularly with longer exposure to the peritoneal fluid, may furnish important data on the influence of bile on *B. typhosus* and similar questions discussed in this paper.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION IN GUINEA-PIG BILE

A series of quantitative tests with the hepatic bile of guinea-pigs that had been collected under oil, conducted in an identical manner to

those reported for the rabbit, demonstrated, when seeded with *B. typhosus*, *B. dysenteriae* and *Vibrio cholerae*, a similar change in the rate of growth and in the subsequent development of antiseptic properties. One set of experiments is chosen in charts 3, 4, and 5, to illustrate the points of importance.

In contrast to our qualitative tests with bile collected unprotected in open tubes, in which most of the selected bacteria were destroyed in 48 to 96 hours, guinea-pig hepatic duct bile collected under oil is a good culture medium for the first 24 to 48 hours of incubation, and develops after this period germicidal properties for *B. typhosus* and *B. dysenteriae*. Bile samples kept exposed to the air or in a desiccator

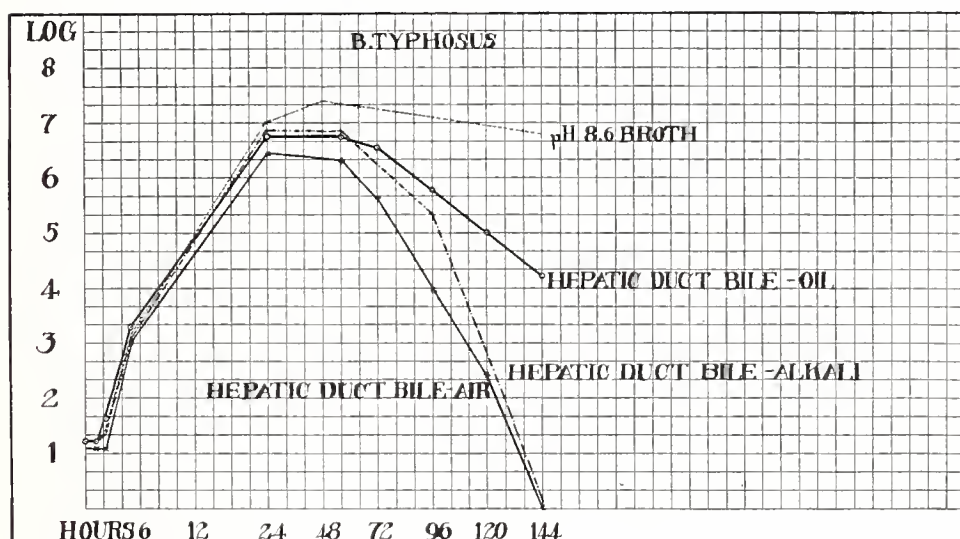


Chart 3.—Rate of growth of *B. typhosus* in the hepatic duct bile of guinea-pigs.

become antiseptic sooner for *Vibrio cholerae* than for *B. typhosus* and *B. dysenteriae*. It is, furthermore, shown that the same bile stratified with oil preserves the growth of *Vibrio cholerae* and also of *B. typhosus*, but does not prevent the development of germicidal substances for *B. dysenteriae*. The disappearance of viable dysentery bacilli occurs simultaneously in the tubes exposed to air or to alkali, or in the desiccator, as it does in the tubes stratified with oil. This event is fairly constant for a number of hepatic duct biles studied, and represents a noteworthy difference from the rabbit biles. It appears rather unlikely that under these conditions the development of germicidal properties is directly connected with a change in the reaction.

This suspicion is supported to a certain degree by the findings made on *Vibrio cholerae*. Chart 5 demonstrates the disappearance of viable spirilla in the tubes exposed to air and in the desiccator on the 96th and 144th hour, respectively. It is recalled that these organisms remain viable in rabbit bile for at least 288 hours, and in the desiccator even longer, at a period of the experiment when the alkalinity has probably reached its maximum (P_H^+ 9.2; titrable — 0.75). The persistence of bacteria rather sensitive to alkalis, like the Shiga dysentery organism in contrast to the alkalophilic *Vibrio cholerae* which disappears rapidly from guinea-pig bile, suggests the coexistence of other factors more potent than the change in the H-ion concentration as the agents respon-

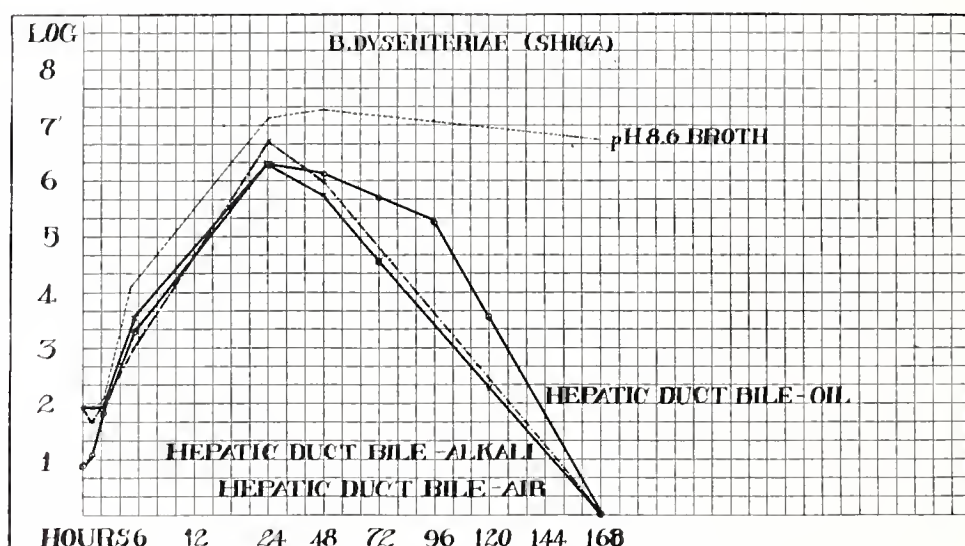


Chart 4.—Rate of growth of *B. dysenteriae* (Shiga) in the hepatic duct bile of guinea-pigs.

sible for the germicidal properties of hepatic duct bile exposed to air. Such substances develop rapidly on standing, when bile is collected in open tubes.

In another experiment bile was collected in open tubes and exposed to air for 4 hours before inoculation with *B. typhosus*. At the same time as a control a sample was stratified with paraffin oil. The sample exposed to air favored the growth for 5 to 8 hours; then germicidal substances produced a progressive disappearance of viable organisms. In 48 hours, sometimes in 24 to 36 hours, such a bile sample was completely sterilized. This experiment explains fully the results of Nichols, who collected the bile in open tubes and kept it at room temperature for more than 3 hours.

It has been repeatedly stated that dilution has little effect on the H-ion concentrations of bile. The tests under consideration are in every respect identical to those reported for rabbit bile with the exception that guinea-pig bile diluted with distilled water produces a more favorable medium than when diluted with saline. The distinct reciprocal relationship between dilution and viability of *B. typhosus* is also clearly demonstrated. The greater the dilution, the longer the persistence of viable organisms. Old and decidedly bactericidal biles, which destroy large numbers of *B. typhosus* in 6 hours, favored the growth and viability of these bacteria even in dilutions of 1:1 or 1:2. Differences in the H-ion concentration could not be recorded by our crude colori-

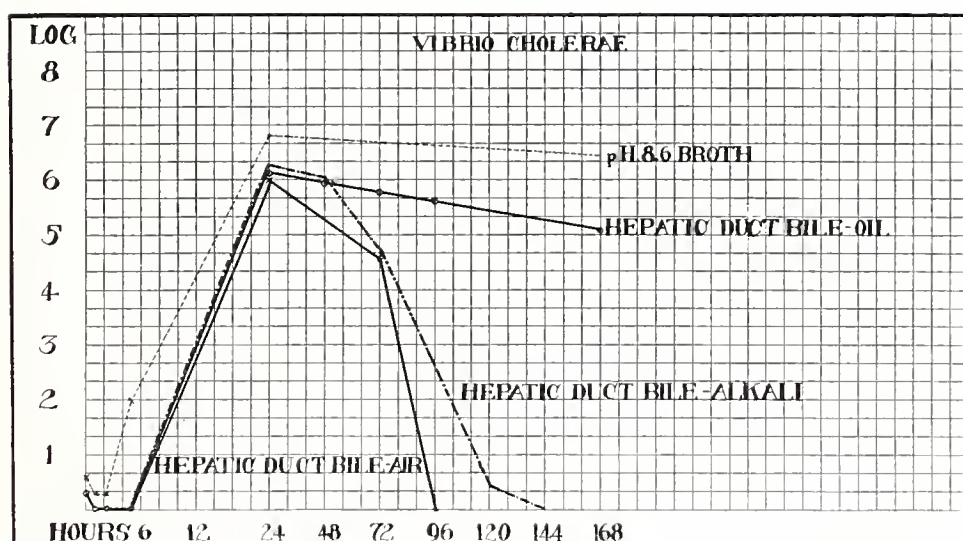


Chart 5.—Rate of growth of *Vibrio cholerae* in the hepatic duct bile of guinea-pigs.

metric method. The addition of 0.25 c c of sterile, heated guinea-pig serum prolonged the existence of viable bacteria in the proportion to the resulting dilution and not as would be expected in accordance with addition of nutritive material and buffers. Irrespective of all the changes brought about by these manipulations, the hepatic bile of guinea-pigs acquired antiseptic properties after a time which depended entirely on the degree of the dilution.

Neutralization with lactic or metaphosphoric acid prolonged the persistence of viable *B. typhosus* for a few days. The results of these experiments were practically identical to those reported for the rabbit and the same interpretation can be applied to the guinea-pig's hepatic duct bile.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION
IN DOG BILE

The rate of growth and the lethal action of hepatic duct and of cystic bile procured from two dogs is shown in chart 6. Both animals showed a marked reflex in the secretion of the duct bile, and received either sodium taurocholate or ox bile during or before the collection of the bile specimens. Dog 17-170 secreted a nongermicidal bile for *B. typhosus* before the injection of sodium taurocholate. The pooled specimens collected after the injection were decidedly antiseptic as is clearly indicated by the curve in chart 6. A large number of typhoid bacilli was destroyed in less than 60 hours. Dog 17-220 was fed ox

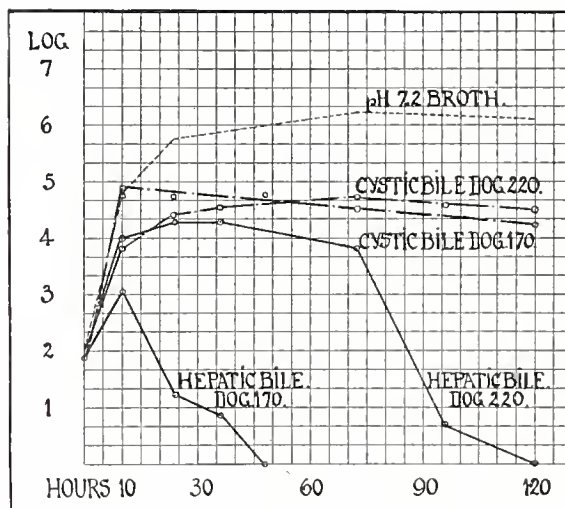


Chart 6.—Rate of growth in and lethal action of hepatic duct and cystic bile procured from two dogs.

bile 4 hours previous to the operation and the collection of the bile specimens. *B. typhosus* grew fairly well for 30 to 40 hours. The bile, however, acquired antiseptic properties and after 120 hours was found to be sterile.

Our observations which confirmed Ecker's,¹⁹ prove the inhibitive and germicidal properties of bile salts on *B. typhosus*. In the course of test-tube experiments with bile salts added to suitable mediums of varying H-ion concentrations, it was noted that a P_{H^+} over 8.0 markedly increased the bacteriostatic properties of bile salts in concentrations of less than 0.5%. A 1% solution of sodium taurocholate in a weak peptone salt solution at a P_{H^+} of 8.4 was strictly germicidal. These

observations have a direct bearing on the explanation of the germicidal properties of the two hepatic duct biles collected from dogs which were treated with sodium taurocholate in order to overcome the reflex inhibition of the bile flow. The familiar cholagogue action of sodium taurocholate given intravenously or by mouth, and the fact that from 40 to 90% of the introduced bile salts are eliminated in 4 to 6 hours (Foster, Hooper and Whipple⁷⁷), immediately suggest a relationship between the increased amount of bile acids and the germicidal properties of hepatic duct bile. The absence of an antiseptic action of cystic bile is explained by the fact that the method of collecting the bile prevented the entrance of hepatic duct bile into this receptacle. These conclusions were confirmed by a quantitative analysis of the bile acids present in the hepatic duct bile of dog 17-170 and dog 17-220. We gratefully acknowledge the assistance received from the late Miss M. G. Foster, who made the determinations of taurocholic acid by the method developed by herself. The elimination of taurocholic acids were:

Dog 17-170 (weight 14.5 lbs.): Before the intravenous injection of 1 gm. of sodium taurocholate the hepatic duct bile contained 8 mg. of taurocholic acid per c.c. In this sample *B. typhosus* grew for more than 10 days. After the intravenous inoculation of 2 gm. of bile salts the pooled bile specimen collected for a period of 5 hours (total 28.8 c.c.), contained 103 mg. per c.c. (or five times the maximum amount ordinarily found in hepatic duct bile or 1% of sodium taurocholate).

Dog 17-220 (Weight 45.25 lbs.): This dog was fed 400 c.c. of fresh ox bile by stomach tube 3 hours preceding the operation and the collection of bile samples. The pooled hepatic bile collected for a period of 4½ hours (total 106.9 c.c.) contained 63 mg. taurocholic acid or over 0.005% of sodium taurocholate.

The bile samples collected from the two dogs possessed, therefore, abnormal amounts of bile acids. In combination with the progressively increasing H-ion concentration, a strongly germicidal medium was produced. The gradual disappearance of viable bacteria in these bile samples is therefore readily explained. The absence of antiseptic properties in hepatic duct bile of some dogs treated with sodium taurocholate was due to a high H-ion concentration or perhaps to an absence of the cholagogue action of the administered bile salts or to both. It is obvious that the explanation offered may not be the only one, and that further investigations alone can furnish the answer to some of the questions which remain unsolved in this preliminary inquiry.

⁷⁷ J. Biol. Chem., 1919, 38, pp. 379, 354.

THE RATE OF GROWTH AND THE SELECTIVE ANTISEPTIC ACTION
IN FRESH AND OLD CYSTIC BILE OF THE OX

Fresh, unheated cystic bile of the ox inoculated with a large amount of typhoid bacilli still contained, after 10 days' incubation, viable bacteria. Fornet,²⁰ Ecker,¹⁹ Pies²³ and others found certain bile antiseptic properties. Quantitative tests were made with fresh unsterilized gallbladder biles collected from beef animals in San Francisco during the months of March and April.

According to chart 7, comparatively small amounts of *B. typhosus* (strain K) in fresh unheated ox bile when stratified with oil grew

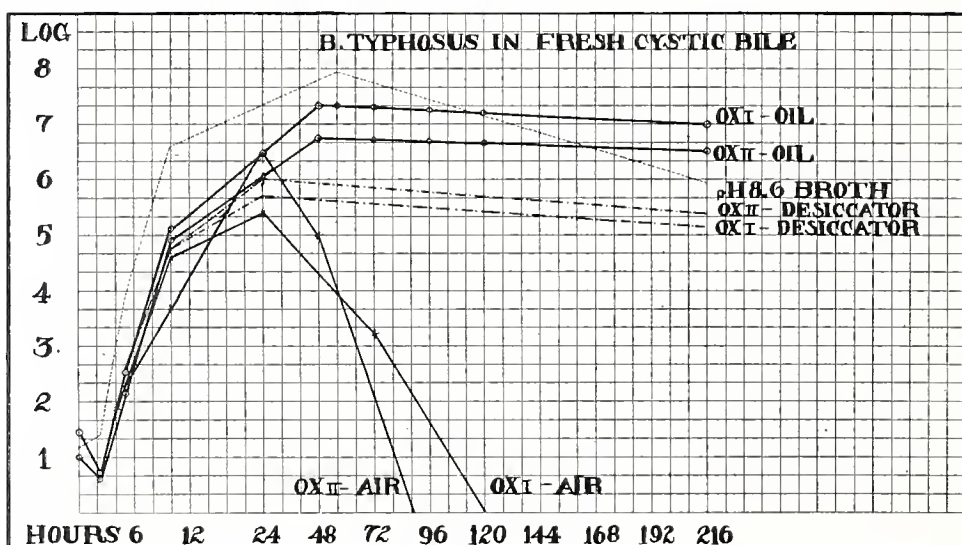


Chart 7.—Rate of growth of *B. typhosus* in fresh cystic bile of oxen.

freely, perhaps not as rapidly as in broth of a P_H^+ of 8.6. Even in the desiccator the development and persistence of viable organisms was fair. Decidedly antiseptic were the same bile specimens (5 c c) when kept exposed to ordinary laboratory air. A 24-hour period of active growth was followed by a progressive disappearance of living *B. typhosus*, and sterility was usually attained between the 72nd and 120th hour. The colorimetric titration gave always P_H^+ readings >8.6 . The reaction changed slowly from a slightly, to a strongly, alkaline one, and regularly reached a lower H-ion concentration than the specimens kept in the desiccator, in which the absorption of ammonia was prevented. On the other hand, samples covered with oil showed occasion-

ally a slight increase in the H-ion concentration from P_{H^+} 8.0 to P_{H^+} 7.6, probably due to the liberation of CO_2 by the cells or to lactic acid formation, the result of bacterial fermentation or destruction of cellular elements on standing. The difference in the behavior of ox bile in comparison to rabbit's and guinea-pig's bile was found in the tubes kept in a desiccator. We are inclined to explain this difference on the basis of a higher H-ion concentration. Furthermore, hepatic duct bile of rabbits has a different composition than cystic bile of oxen, and changes in the H-ion concentration may produce more potent germicidal forces in the former than in the latter. The varying composition of cystic ox bile intimately connected with the food of the animal is of greatest importance. It explains, in all probability, the varying results reported by different investigators.

The selective action of fresh unheated ox bile on *Vibrio cholerae* and *B. dysenteriae* Shiga was also studied. According to Schöbl, ox bile is a good culture medium for *Vibrio cholerae*. There may be a slight inhibitive action and the indication of a lag. The growth may be less heavy than in a plain salt-free peptone broth of a P_{H^+} of 8.6. *B. dysenteriae*, however, cannot thrive in ox bile exposed to air; invariably 1500 organisms or less were destroyed in from 12 to 48 hours. This corresponds with the findings of Marbais.⁷⁸ Some specimens stratified with oil sterilized themselves of *B. dysenteriae* in 168 hours. In all three samples in which this took place, a shift of the H-ion concentration above P_{H^+} 8.4 was noted and reached in the light of the observation of Cohen and Clark⁷⁹ the border of the P_{H^+} zones. Reproduction and viability of *B. dysenteriae* Shiga is completely impaired. Tests with Flexner bacilli produced similar results. Occasionally, bile specimens were found which permitted an indefinite growth of these organisms, as Flu¹² has recorded for beef biles freely exposed to air.

Ox bile kept at room temperature exposed to diffuse light or in the ice-chest under oil or exposed to air and heated at 60 degrees for 30 minutes or autoclaved, produced a medium with a peculiar action on *B. typhosus*, which is illustrated by chart 8. In the experiments on which this chart was based, 5 c c samples of bile were inoculated with about 500 living *B. typhosus*. Antiseptic properties reduced this number materially in from 1 to 10 hours. This period of destruction

⁷⁸ Compt. rend. Soc. Biol., 1913, 81, p. 1136.

⁷⁹ J. Bacteriol., 1919, 4, p. 409.

of viable organisms varied with the different bile samples. The degree of germicidal action was occasionally so marked that a 0.1 c c specimen was found to be free from viable *B. typhosus*. If a small number of bacteria was seeded in such biles, a mortality of 100% or a complete sterilization might ensue, confirming Fornet²⁰ and Pies.²³ As a rule, however, the germicidal action on the part of the medium was followed by a pronounced more or less varying "lag" which in turn was succeeded by a gradual multiplication of the apparently seriously injured cells. Progressive proliferation of *B. typhosus* extended over 7 days, and not only reached, but sometimes surpassed the total number of viable organisms present in the broth control. This characteristic

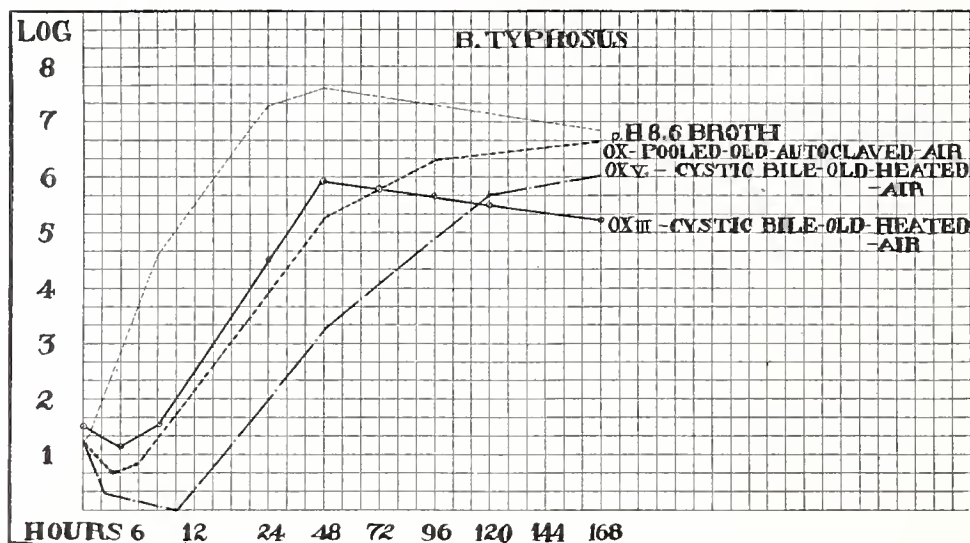


Chart 8.—Rate of growth of *B. typhosus* in bile of an ox.

behavior of old heated cystic bile of the ox as a medium for *B. typhosus* is apparently not connected with the H-ion concentration of the secretion. Not only were the specimens acid to litmus and phenolphthalein, but the P_{H^+} never decreased below 8.2. In the course of the experiment, probably as a result of the growth of *B. typhosus*, it even increased to P_{H^+} 8.0 or even 7.6. From the standpoint of reaction, the ox bile became therefore more and more suitable. It is not unlikely that simultaneously a new strain was gradually generated with an acquired resistance to the antiseptic and bacteriostatic properties of the old bile. This new strain may in part be responsible for the slow but progressive

proliferation after the 24th hour. A limited number of observations made by successive transfers of *B. typhosus* through biles of the same animal support this explanation.

As repeatedly emphasized, our study of ox bile was primarily undertaken to correlate the findings made with biles of other laboratory animals and to demonstrate conclusively that any bile on standing in a test tube undergoes changes which produce a germicidal, sometimes even a selective action, on a variety of bacterial types. Heating, filtering and other manipulations only enhance these properties and produce finally in vitro a medium of such complexity that it is impossible even to surmise how it acted in its natural environment, the gallbladder. The

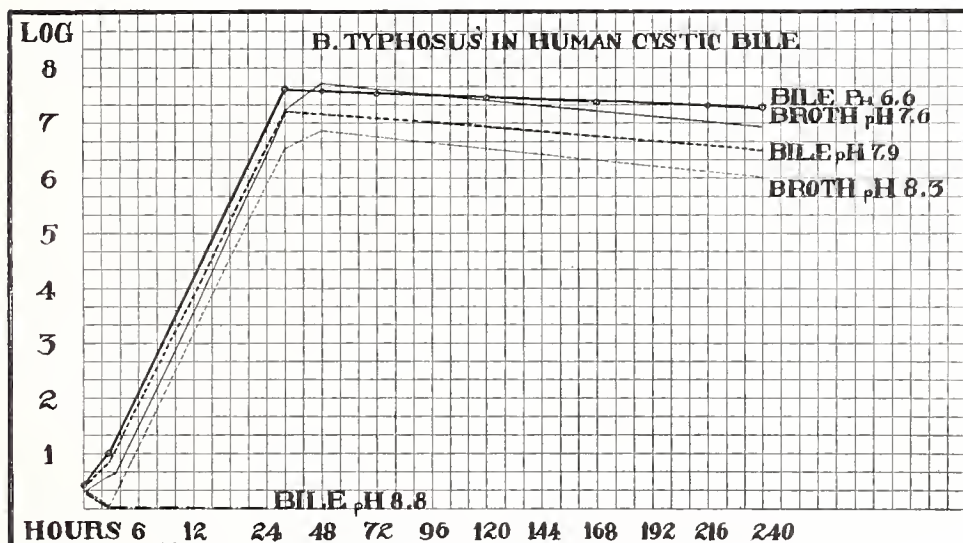


Chart 9.—Rate of growth of *B. typhosus* in human cystic bile.

recognition of this fact, which does not apply to the study of bile alone but to many other body secretions, is frequently ignored by the majority of bacteriologists.

THE RATE OF GROWTH OF *B. TYPHOSUS* AND *VIBRIO CHOLERAE* IN HUMAN CYSTIC BILE AT VARYING H-ION CONCENTRATIONS

Human cystic bile collected at necropsy or during laparotomies or cholecystectomies is, as a rule, a good medium for the development of a variety of bacteria which can remain viable for 60 days and longer. (Toida²⁷ and others.) Hepatic fistula bile proves a good medium for every micro-organism inoculated. Some investigators, Pies,²³ Miecz-

kowski,³² Hirokawa,³⁹ and Toida²⁷ report, however, a marked degree of initial inhibition for a number of samples studied. We were unable to find such a specimen and can therefore not express an opinion on the nature of this bacteriostatic action. It is rather remote to suspect a change of reaction to be also responsible for this "lag," as was the case for the various other biles investigated. Our observations have demonstrated that the fairly alkaline or neutral human cystic bile changes slightly when standing exposed to air.

Fresh human bile was tested to compare its behavior with that in which the H-ion concentration had been previously artificially changed. The growth curves are shown in charts 9 and 10.

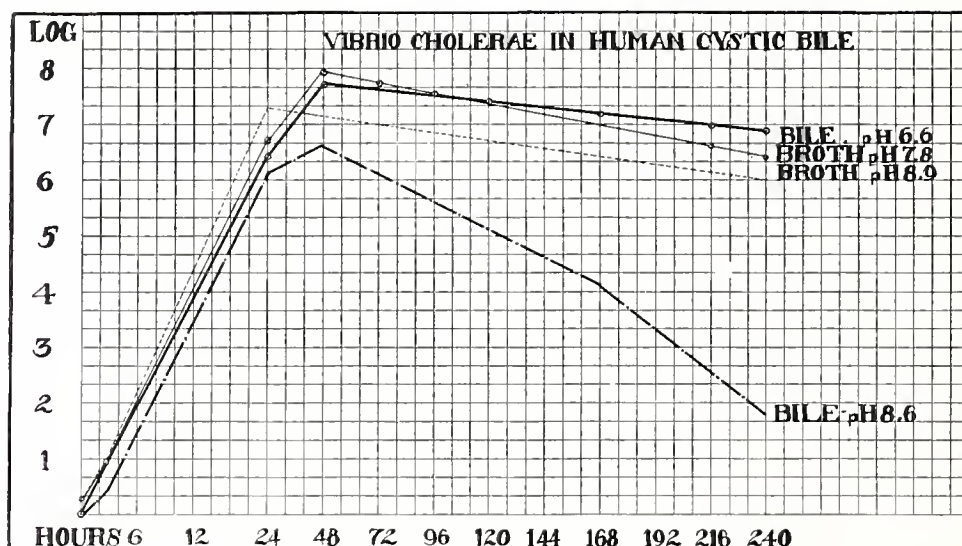


Chart 10.—Rate of growth of *Vibrio cholerae* in human cystic bile.

It is evident that the proliferation of *B. typhosus* and *Vibrio cholerae* is only impaired or even completely suppressed at a H-ion concentration which in all probability never occurs in the human gallbladder. Human cystic bile must be considered a suitable medium for the viability of the two organisms; at least, in comparison with ox or rabbit bile the vigor of the bacterial growth is noteworthy. The sample chosen for the determination of the growth curves shown in the charts was derived from a gallbladder with a slight stasis as a result of fasting. The growth-favoring influence of starvation on the hepatic duct and cystic bile of rabbits has been previously mentioned. It was also pointed out that

stasis favors gallbladder infection and therefore the carrier state. The growth curves presented here may therefore be of practical significance and suggest a possible connection between suitability of cystic bile as a medium for *B. typhosus* and for *Vibrio cholerae*; this may also explain the gallbladder carrier state. Variability in the composition, for example, changes in the proportion of taurocholic to glycocholic acid, and perhaps the reaction, influence the selective growth of different bile samples, as is well illustrated by the observations of Hirokawa,³⁰ Toida,²⁷ and others; but only a large series of tests will demonstrate beyond a doubt the actual importance and influence of the bile as such in the human typhoid carrier state.

SUMMARY

Hepatic duct bile of rabbits collected in open tubes and seeded with various pathogenic and nonpathogenic intestinal bacteria exhibits selective germicidal properties. The organisms of the typhoid-dysentery-paratyphoid group remain viable for 72 to 96 to 120 hours, and those of *Vibrio cholerae* and staphylococcus for over 7 to 10 days. A small number of bacteria is more readily destroyed than those of a heavy inoculum. Base-forming diets produce, on the average, bile specimens in which the time rate of lethal action is slightly shorter than in those procured from animals fed on acid-forming diets. True gallbladder bile specimens collected from the same animals and tested under the same experimental conditions fail to exhibit germicidal properties in the chosen time interval of 10 days.

Guinea-pigs' hepatic duct and cystic bile exhibits under identical experimental conditions greater germicidal action against the same number and the same type of bacteria than rabbit-liver bile. *B. typhosus*, *B. paratyphosus*, and *B. dysenteriae* are usually destroyed in from 24 to 48 hours; even *Vibrio cholerae* cannot survive a period of 120 hours. Fresh cystic bile of guinea-pigs, accidentally suffocated, was not germicidal for 400 hours.

Gallbladder bile of dogs kept on a mixed diet is apparently indifferent for a variety of bacteria; the inoculated organisms remain viable for over 10 days. Hepatic duct bile of the same animals treated with bile salts as cholagogues may be germicidal and destroy *B. typhosus* in from 24 to 120 hours.

Qualitatively at least the hepatic duct bile of cats, goats, rats, monkeys and man, as well as the gallbladder bile of oxen, sheep and pigs

favor the viability of *B. typhosus*. In such bile specimens inoculated with a large number of bacteria, living germs could be demonstrated in open tubes after 10 days of incubation at 37 C. Typhoid bacilli show a high degree of viability in human hepatic duct and cystic bile. *Vibrio cholerae* may be destroyed in 8 days. We failed to observe in one case of human biliary fistula after the institution of an extensive alkaline therapy, the development of germicidal properties against *B. typhosus*.

Nonpurulent gallbladder biles of typhoid-infected or carrier rabbits, guinea-pigs, monkeys and man behave either like hepatic duct bile specimens or like true cystic bile samples when removed from the viscus and exposed to air. Purulent specimens from rabbit or man, when incubated at 37 C., may show viable bacteria for over one month. The physical consistency, the cellular contents, and the duration of the carrier state govern the persistence of living *B. typhosus* in such specimens.

The germicidal properties of hepatic duct bile of rabbits and of guinea-pigs is not due to bacteriolysins or a "substance sensibilatrice." Such specimens never contain agglutinins. According to our observations, about 25% of the gallbladder bile specimens derived from laparotomized immune rabbits may contain agglutinins in a dilution of over 1:50. Recovered carriers may give clumping in dilutions above 1:100. The biles of guinea-pigs are always found to be free from agglutinins. One-half of the biliary secretions collected from "carrier" rabbits agglutinate *B. typhosus* in dilutions above 1:100. The degree of the inflammatory process and the stasis in the gallbladder are probably some of the factors which are conducive to the presence of agglutinins.

Quantitative tests with hepatic duct bile of rabbits collected under oil but tested in vitro exposed to air or to alkali have shown that *B. typhosus* grows, following a short lag, for at least 24 hours. However, in the next 24 to 48 hours, the bile acquires, probably on account of changes in the H-ion concentration (escape of CO₂, absorption of ammonia), antiseptic properties which lead, irrespective of the diet of the animal, to complete disinfection of the fluid in 96 hours. In hepatic duct bile stratified with paraffin oil and in cystic bile *B. typhosus* shows a high degree of viability. The *Vibrio cholerae* behaves similarly. These facts disprove, in our opinion, the theory that rabbit bile is germicidal in vivo.

Old, sterile bile kept at room temperature or in the ice-chest is germicidal for *B. typhosus* and *B. dysenteriae* Shiga after 24 hours' exposure at 37 C. Such bile specimens can be rejuvenated by saturation with CO₂ and subsequent stratification with oil. Neutralization of germicidal bile may produce temporarily, at least, a fluid in which a variety of bacteria can develop for from 1 to 8 days. The addition of strong acids alters the bile in many respects. Feeding or the injections of alkalis and acids, which produce a state of experimental alkalosis or acidosis, do not materially influence the germicidal properties of the hepatic duct bile of rabbits.

The H-ion concentration of the hepatic duct bile is only indirectly responsible for its antiseptic properties. Dilution of this secretion with distilled water in the proportion of 1:1 or even 1:2 fails to change the reaction but alters the concentration of the bile salts. It prolongs the persistence of viable bacteria for several days in comparison to the undiluted control tubes. Sterile serum acts in the same manner as distilled water. The growth-enhancing influence is in all probability not caused by the addition of nutritive material or "vitamines." The hepatic duct bile of hypercholesterolized rabbits develops germicidal properties after the same time interval as was observed for normal rabbits.

Antiseptic rabbit bile sealed in collodion sacs and placed in the peritoneal cavity of rabbits was found to be an excellent medium for the growth of *B. typhosus*.

Fistula bile of guinea-pigs collected under oil is a good medium for *B. typhosus*, *Vibrio cholerae* and *B. dysenteriae*. On standing exposed to air it changes its reaction and acquires germicidal properties which destroy the viable organisms in from 96 to 168 hours after inoculation and incubation at 37 C. One strain of an alkalophilic *Vibrio cholerae* was apparently more readily destroyed than several strains of *B. typhosus* and *B. dysenteriae*. The initial "lag" in fresh bile is slight and of short duration. Bile collected in open tubes or exposed for 4 to 24 hours to air becomes germicidal in a shorter time interval than fresh bile. The inoculated bacteria are destroyed in 24 to 48 hours or even less. Such biles, when diluted with distilled water or saline, favor the persistence of viable *B. typhosus* irrespective of the H-ion concentration. A distinct reciprocal relationship exists between dilution and viability of bacteria. *B. typhosus* will remain viable for over 10 days when the hepatic duct bile of the guinea-pig is diluted in the proportion of 1:9.

Fistula bile collected from dogs which received ox bile by mouth or sodium taurocholate intravenously in order to counteract the post-operative reflex inhibition on the biliary system, may contain more than 0.005% of taurocholic acid per c c. The salts of this acid are strongly antiseptic in an alkaline medium. As the hepatic duct bile of the dog also becomes alkaline (P_H^+ 8.2-8.4) on standing exposed to air, it is not surprising to observe the development of germicidal properties for *B. typhosus* in such specimens.

Fresh unheated sterile cystic ox bile develops, on incubation and exposure to air, germicidal properties. A certain number of the viable *B. typhosus* and *B. dysenteriae* are prevented from multiplication, and a large number of bacteria is actually destroyed in the first two hours after transference to the ox bile medium. This period is followed by a "lag" of varying length, which in turn is succeeded by a rapid proliferation. The maximum development is reached on the 12th or 48th hour for the specimen exposed to air. Progressive destruction, however, sterilizes these specimens in from 12 to 120 hours. The time required depends entirely on the bile specimen. The *Vibrio cholerae* is less seriously injured than typhoid and dysentery bacilli. Sterilization of the samples exposed to air or alkali has not been noted. Ox bile prevented by stratification with oil from giving off CO_2 or from absorbing ammonia in a desiccator does not become germicidal. Old, heated or unheated ox bile acts in a characteristic manner on *B. typhosus*. Immediately following the inoculation of the bile a marked destruction of viable organisms is recorded. This germicidal action may lead to complete sterilization of the bile, or is followed by a pronounced lag, which in turn is succeeded by a slow, but progressive, multiplication of the bacteria. The maximum growth may only be attained after 7 days, and the growth curve may at this time not only reach but even surpass the one of the broth control. This action of the bile is entirely independent of the reaction of the secretion. Our experiments confirm those of other workers, and they should prove conclusively that cooked ox bile is unsuitable for the primary isolation of *B. typhosus* from tissues or secretions. Mixed with peptone and glycerol it is, on account of its hemolytic properties, a good substratum for typhoid blood cultures.

The vigor of the bacterial growth in an average human cystic bile is noteworthy. Proliferation can be influenced by artificially reducing the H-ion concentration. Complete suspension of growth, however,

only occurs at a P_H^+ which in all probability never occurs in the human body. The study of a large series of human cystic biles is advocated to determine the actual importance and influence of the secretion as such on the human typhoid carrier state. The observations reported in this paper have in our opinion demonstrated one outstanding fact, namely, a test-tube experiment may be simple and reveal a great deal of information, but it does not always tell the truth and may be most misleading to the pathologist.

THE INFLUENCE OF THE H-ION CONCENTRATION ON THE GROWTH OF *B. TYPHOSUS* IN MEDIUMS CONTAINING BILE OR BILE SALTS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VIII.

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In the preceding paper it was demonstrated that bile, particularly hepatic duct specimens, acquired germicidal properties when exposed to air. Comparative tests indicated that this effect was connected with a lowering of the H-ion concentration. However, it was not entirely evident in what manner this change in the reaction, alone or in combination with the chemical elements of the bile, had acted as the germicidal factor. A few experiments with bile specimens and bile salts at varying H-ion concentrations suggested themselves. These tests, primarily undertaken to study the rate of growth of *B. typhosus* and its generation time, furnished data which indicated that a low H-ion concentration inhibited the growth-stimulating properties of the bile and its salts and rendered such a medium germicidal.

With the exception of Meyerstein,¹ who states that the growth of *B. coli* and *B. typhosus* is abundant only when the mediums containing bile salts are neutral or slightly acid to litmus, recent reports on the subject of ox bile as a culture medium fail to mention the importance of the reaction. Ecker² and Salter³ use neutral or slightly acid mediums which, according to the findings of Meyerstein, are the optimum reaction. Such a favorable reaction is, according to our tests, rarely encountered with the usual ox bile samples obtained in a fresh state from abattoirs, and sterilized while warm. It is quite obvious that the reports of various investigators dealing with the nutritive value of bile for bacteria must be more or less at variance on account of their failure to adjust the reaction of bile or bile salt mediums. Moreover, the proper-

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¹ *Centralbl. f. Bakteriol., O.*, I, 1907, 44, p. 434.

² *Jour. Infect. Dis.*, 1918, 22, p. 95.

³ *Ibid.*, 1919, 24, p. 260.

ties of sodium taurocholate or glycocholate and their decomposition products are not accurately known. On the other hand, it is not our intention to overemphasize the reaction of the bile salt mediums, but in connection with the experimental pathologic studies reported in the preceding papers, it appeared to us as one of the important factors among the many dealing with the so-called antiseptic effect of bile. In order to obtain striking contrasts, two reactions, namely, P_H 7.0 and P_H 8.2 to P_H 8.4 were chosen.

Technic.—One strain of *B. typhosus* (K) was used throughout the entire series of experiments. Transplants were kept on peptic digest agar. Previous to an experiment it was transferred for a few days to 1% ox bile salt-free veal broth. For each test a 18-24 hour old culture in this medium was used.

The salt-free veal broth was prepared in the same manner as stated in the second paper of this series. The 0.01% peptone solution, which was suggested by the work of Meyerstein, was made by dissolving 0.02% "Difco" peptone in distilled water and adding 20 c c of a phosphate mixture of known H-ion concentration to each 100 c c of medium. This mixture was distributed in 30 c c amounts in Pyrex Erlenmeyer flasks; sterile, filtered or unfiltered bile, or any other bile product to be studied was added and the total volume was made up to 50 c c. Sterilization was carried out in live steam on three consecutive days.

A 0.01% peptone solution, according to Meyerstein, is supposed to maintain the life of the organisms and yet not furnish enough nutritive material to permit a vigorous growth of *B. typhosus*. Hence any inhibitive or stimulating properties possessed by bile samples or their salts would be definitely demonstrated.

Several fresh samples of ox bile (12-14 cystic bile specimens) were filtered through paper and sterilized at 15 pounds' pressure. Sterily collected hepatic duct bile samples of several rabbits were pooled and added without sterilization to the medium. Some experiments were also carried out with bile derived from a single gallbladder of the ox. Desiccated, "Difco" ox-bile was dissolved in distilled water and the concentrated solution added to the basic nutritive solution. This preparation is only slightly soluble in distilled water; a 1% solution forms an appreciable precipitate in the neutral medium. The latter was not filtered from the test medium, but care was taken to rotate the flasks thoroughly to insure even distribution of the sediment before samples for plating were removed. The purified bile salts were prepared by Drs. Foster and Hooper from dogs' or pigs' bile and have been tested by them in their studies on the metabolism of bile acids.

Fifty c c of medium were seeded with 0.5 c c of a 1:10,000 dilution of a young culture of *B. typhosus* in 1% ox bile salt-free veal broth. The flasks were warmed to 37 C. before inoculation and kept incubated at this temperature in an electrically controlled water bath. The determination of the number of organisms present at various periods of growth were made by plating in peptic digest agar (P_H 7.0). This medium was prepared in large quantities to insure uniformity in composition. Duplicate and often triplicate plates were poured with dilutions made in sterile salt solution. The plates were counted after 48

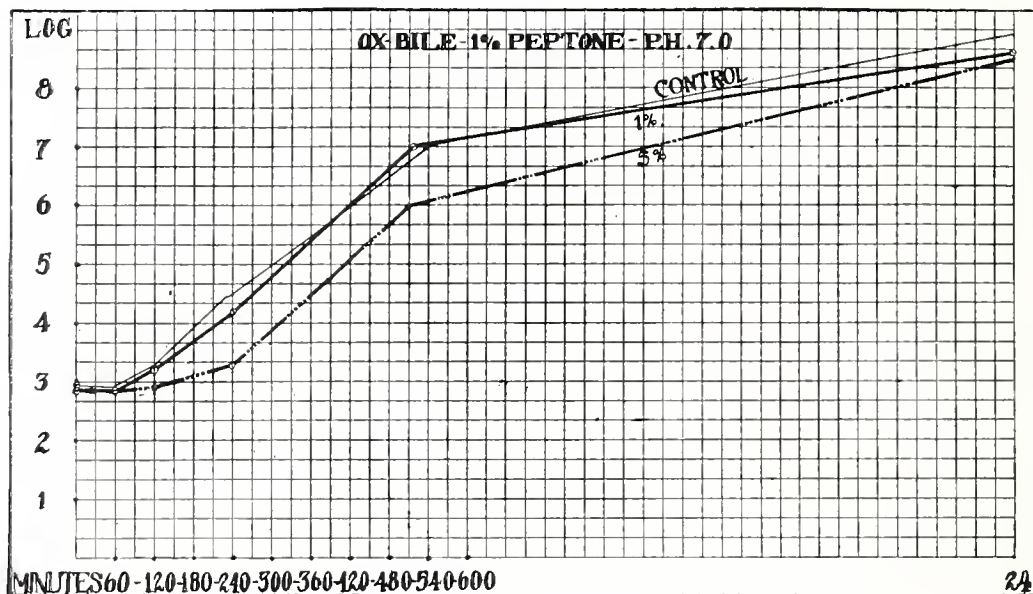


Chart 1.—Rate of growth of *B. typhosus* in filtered sterile ox bile in 1% peptone-phosphate solution, pH 7.0.

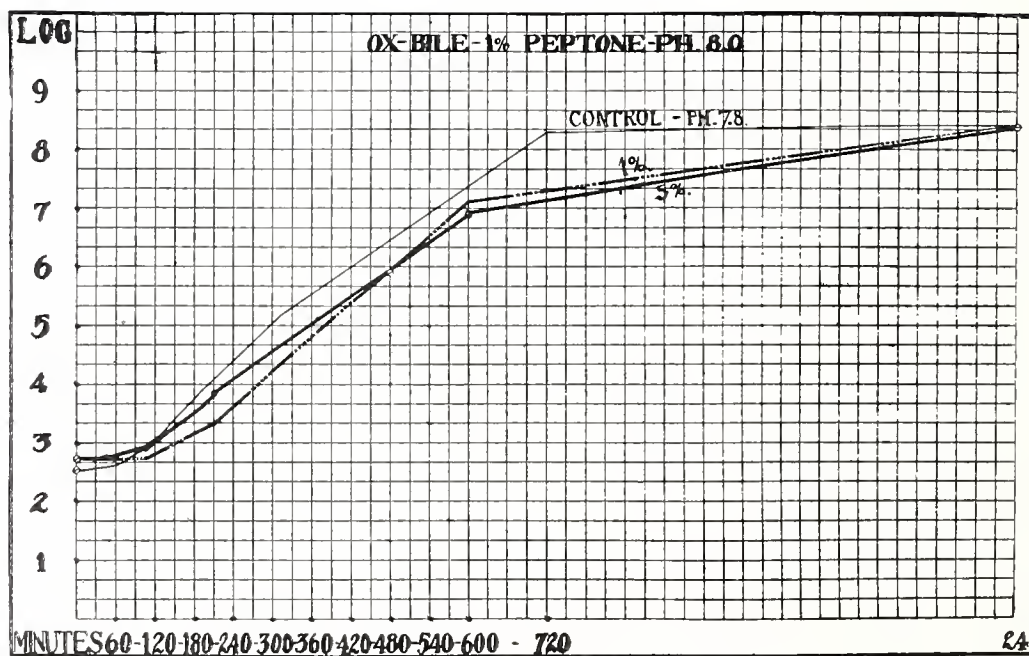


Chart 2.—Rate of growth of *B. typhosus* in filtered sterile ox bile in 1% peptone-phosphate solution, pH 8.0.

hours' incubation at 37 C. Only plates which showed more than 25 or less than 250 colonies were considered accurate. Each experiment was repeated, and only the results in which no marked changes in the P_H reaction of the test medium occurred were noted. Representative data of such series are chosen for discussion of the individual experiments.

In the charts the logarithms of the viable organisms per 1 c c of medium are plotted as ordinates against time intervals (expressed in minutes) as abscissae.

Exper. 1.—Filtered sterile ox bile in 1% peptone-phosphate solution was seeded with *B. typhosus* and kept in water bath at 37 C.

This experiment clearly demonstrates that ox bile in low concentrations, namely 1 and 5% solutions, added to a suitable nutritive substratum, is distinctly inhibitive for *B. typhosus*. These observations confirm those recently published by Ecker. The growth curves of a 1% solution run below those of the control mediums throughout the entire period of the experiment. A 5% solution is decidedly depressant on the development of *B. typhosus*, a fact which is indicated by a distinct lag extending over 80 minutes. The final growth is, however, not materially influenced; the bile-curves show a tendency to reach the same level as the control-curve. Furthermore, the reaction of the medium does not materially alter the rate of growth, as is evidenced from the general behavior of the two curves. The minor differences in the character of the curves may be safely attributed to technical irregularities.

It was quite obvious that a 1% peptone solution was unsuitable to demonstrate conclusively the inhibitive or even the germicidal properties of bile. The available nutritive substances permitted a good initial growth, and the subsequent adaptation to the antagonistic forces of the bile produced the results clearly demonstrated in the curves. Identical observations were made by Pies.⁴ The suggestion of Meyerstein to use a 0.01% peptone solution was therefore followed. It was unfortunate that our experiments, which were intended to retain the initial reaction constant, necessitated the addition of a phosphate mixture. A peptone-phosphate solution is, in contrast to Meyerstein's Witte's peptone solution, a fair culture medium, as the figures in the table clearly demonstrate.

It is evident from the table that the claims of Meyerstein were not confirmed. In several tests a distinct increase of the inoculated *B. typhosus* took place after 24 hours' incubation. The multiplication

⁴ Arch. f. Hyg., 1907, 62, p. 107.

was not as marked as in a "Difco" peptone solution, or in the same solution buffered with phosphates. The phosphate mixture at a P_H 7.0, kept *B. typhosus* alive or stimulated a slight growth. It is, therefore, advisable to test in such a solution the substances which are suspected to exert an inhibitive or germicidal effect on bacteria. For the graphic demonstration of the influence of the reaction on the action of bile salts a 0.01% "Difco" peptone solution with phosphates reproduced the nutritive value of the bile more accurately than the phosphate mixture alone. This conclusion was justified by the numerous successful tests to be reported.

GROWTH OF *B. TYPHOSUS* IN PEPTONE AND PEPTONE-PHOSPHATE MIXTURES P_H 7.0

	Number of Organisms Immediately After Inoculation per C c	Number of Organisms After 24 Hours per C c
Phosphate mixture alone.....	410	860
Difco peptone, 0.01% solution.....	1,600	13,000,000
Difco peptone, 0.01% solution + phosphate mixture	1,600	34,000,000
Witte's peptone, 0.01% solution.....	1,600	1,350,000
Witte's peptone, 0.01% solution + phosphate mixture	1,680	1,560,000

Exper. 2.—Filtered sterile ox bile in a 0.01 "Difco" peptone-phosphate solution, P_H 7.0 and P_H 8.2, was seeded with *B. typhosus*. Incubation occurred at 37 C. Four tests were made.

At a reaction of P_H 7.0, ox bile in quantities of 1% or less was decidedly stimulating for *B. typhosus*. Even the usual lag was entirely absent, and the figures obtained for the final growth exceeded the one for the control flask. Five, 10 and 30% additions of ox bile were not only inhibitive, as indicated by the prolonged lag, but were also depressing the growth curve in general. Attention is called to the inhibition of the growth for at least 5 hours in the 10 and 30% ox bile-peptone solutions. Moreover, in contrast to the 1% peptone solution, the development of *B. typhosus* in a 0.01% peptone medium was slow but progressive, and the curve failed to show the steep rise in the first 8 hours so clearly noted on charts 1 and 2.

At a P_H 8.2, ox bile, even in concentrations below 1%, was distinctly inhibitive. The initial lag for 10 and 30% solutions was extended over a period of from 8 to 9 hours. Furthermore, the final growth as a whole was considerably below the one noted for the medium of a P_H 7.0. In many respects the general character of the 30% ox bile curve corresponded to the one established in the preceding paper (chart 8) for fresh unsterilized ox bile of a reaction of P_H 8.2-8.6. These findings.

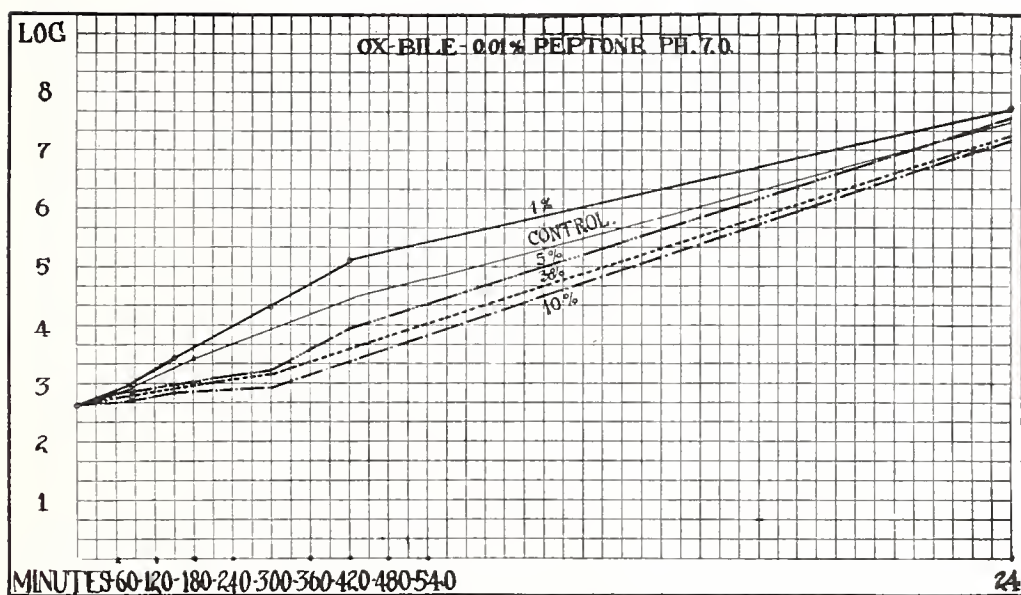


Chart 3.—Rate of growth of *B. typhosus* in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 7.0.

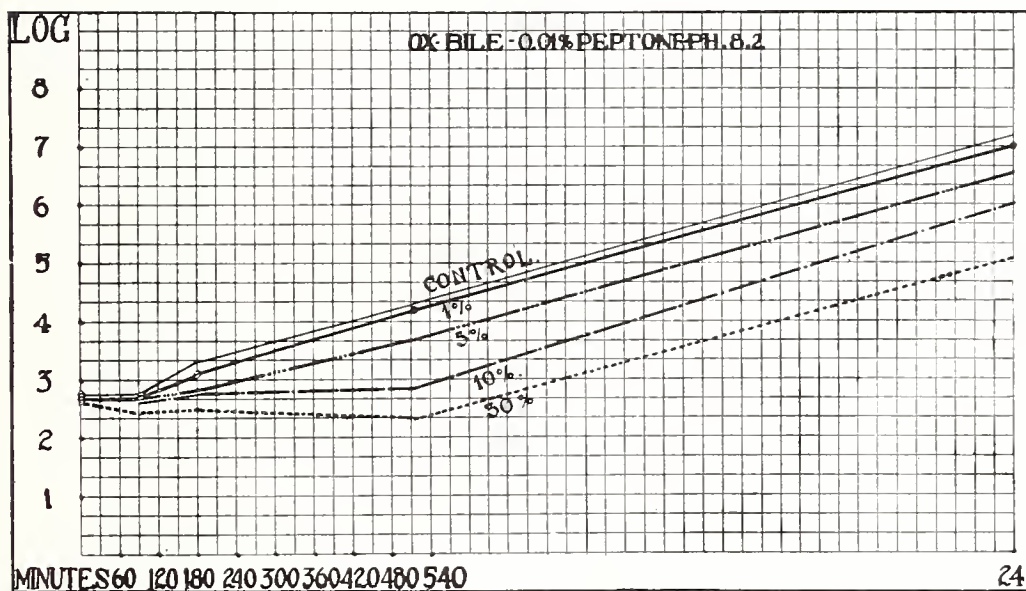


Chart 4.—Rate of growth of *B. typhosus* in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 8.2.

demonstrated that ox bile at a P_H 8.2 inhibits a large proportion of the viable cells.

The nature of the inhibitive substances in ox bile is for the present omitted from consideration. Whether the specific stimulating effect of this secretion in small concentrations at a P_H 7.0 is merely the result of additional nutritive material or due to certain definite substances, is difficult to say. It is recalled that Salter found bile salts in small quantities to be growth promoting for *B. coli*. Bile in low concentrations can therefore act in a similar manner on all members of the colon typhoid-paratyphoid group.

Expcr. 3.—"Bacto" desiccated ox bile in 0.01% "Difco" peptone-phosphate solution, P_H 7.0 and 8.2, was seeded with *B. typhosus*. Incubation occurred at 37 C. Two experiments were made.

The influence of the reaction on the effect of bile salts as culture mediums for *B. typhosus* was well illustrated by this experiment. Thus it was shown that neutrality supported the growth producing properties of a low concentration of bile salts, while a strongly alkaline reaction rendered the same substances not only inhibitive, but germicidal. At P_H 7.0 a 30% "bacto"-bile salt medium was slightly inhibitive and a 0.5 and a 1% solution were stimulative. The curve for a 2% bile salt medium was not shown in the chart. It covered the one for the control medium.

At a P_H of 8.2 a 1%, even a 0.2%, solution of desiccated bile was germicidal for *B. typhosus*. The rate of lethal action was shortened by the concentration of the medium in biliary elements or, in other words, the more concentrated the fluid, the greater was its effect on the viable cells. It was furthermore shown that the germicidal action did not manifest itself for at least 3 hours in the 0.2 and 0.5% bile salt medium. There were indications that for this time period the inoculated *B. typhosus* not only remained viable, but proliferated to a slight degree. This particular phenomenon was not absolutely constant and certain unknown factors, possibly similar to those noted by Cohen and Clark, produced slight variations. The curves shown in charts 5 and 6 represent the general behavior of *B. typhosus* in bile salt mediums at different H-ion concentrations, and find their analogues in some of the charts made from determinations on fresh, undiluted animal bile, discussed in the preceding paper.

Expcr. 4.—Sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution was seeded and incubated as usual.

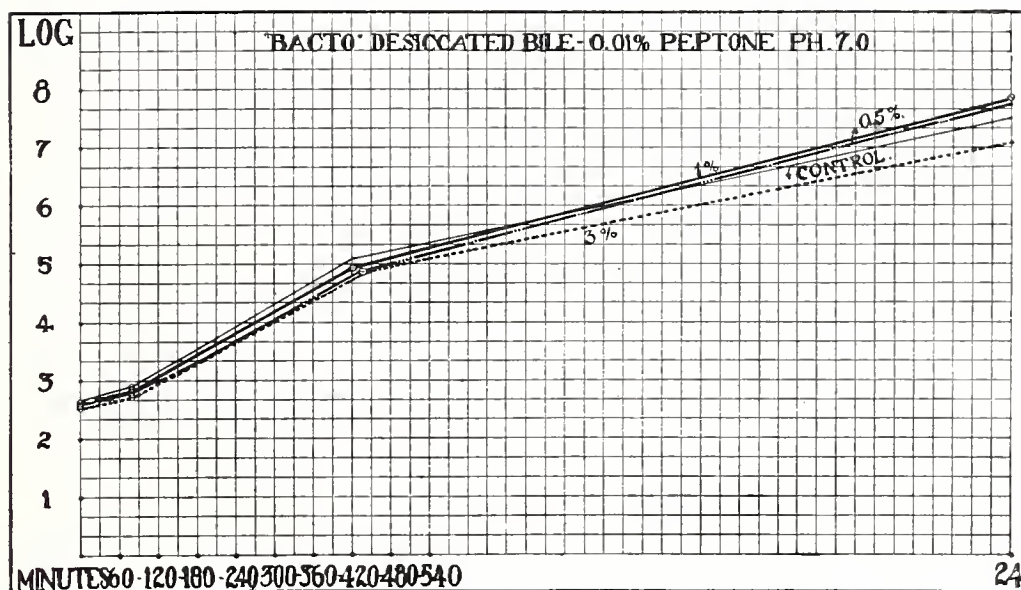


Chart 5.—Rate of growth of *B. typhosus* in "bacto" desiccated ox bile peptone-phosphate solution, PH 7.0.

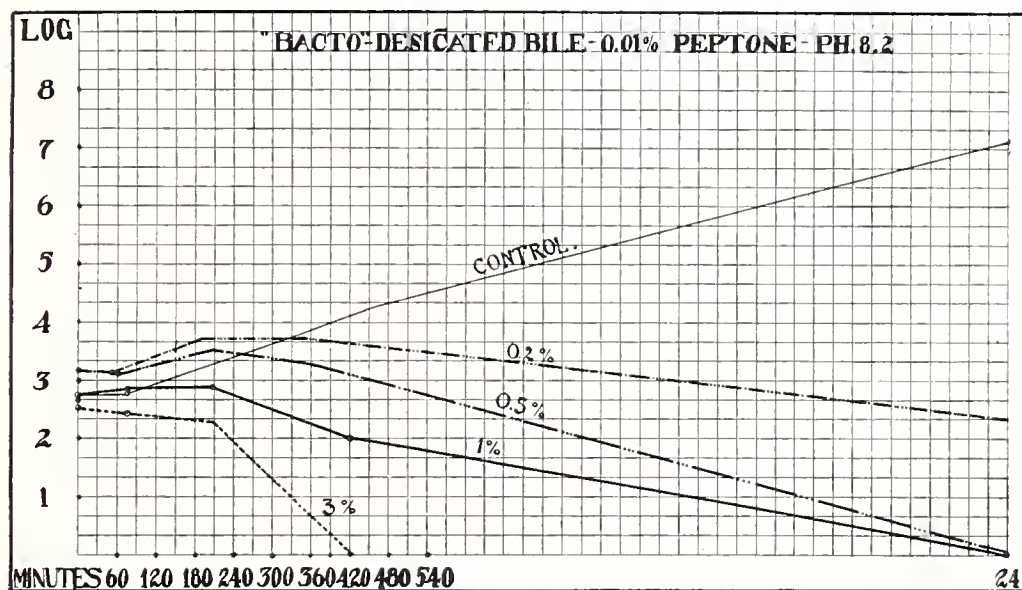


Chart 6.—Rate of growth of *B. typhosus* in "bacto" desiccated ox bile peptone-phosphate solution, PH 8.2.

The curves presented in charts 7 and 8 confirmed our previous observations with undiluted hepatic duct bile of the rabbit exposed to air for several days. However, the data revealed some interesting properties which were not evident in the tests conducted with undiluted bile. Rabbit bile was not only less inhibitive than ox bile in a neutral medium, but must be considered stimulating and growth enhancing. Additions of bile (not exceeding 10%) invariably produced better development of *B. typhosus* than a peptone-phosphate mixture alone. The paradoxical behavior of the 5 and 10% solutions of rabbit bile was constant in the 2 experiments conducted. For the present we are unable to offer an explanation for the observations, but desire to call attention to a similar observation recorded by Meyerstein. In the course of some tests with purified crystallized ox bile, this worker noted that *B. typhosus* and *B. coli* grew well in a 10% solution of a mixture of sodium taurocholate and sodium glycocholate (40 and 60%), while lower concentrations (1 to 5%) completely inhibited proliferation of these organisms. He states that this action of the bile salts was not recorded with the desired regularity to permit a final conclusion. In the light of the experiments conducted with pure bile salts, it is suspected that these paradoxical results are due to the glycocholic acid or its salts. Further tests are, however, necessary to prove this contention in a conclusive manner.

The general tendency of the growth curve at P_H 7.0 also indicated that the typhoid bacillus adapted itself more readily to the elements contained in the rabbit bile than to those of ox bile. The initial lag was comparatively short and between the 2nd and 6th hour the proliferation was very active in the 5 and 10% solutions. The final growth at the end of 24 hours, however, did not materially exceed the one reported for ox bile.

An alkaline reaction of P_H 8.4 affected the inoculated bacteria in a manner already known. In the 2 experiments the results differed somewhat from each other, but the curves shown in chart 8 present the average behavior of *B. typhosus* in rabbit bile at P_H 8.4. Moreover, the 5% solution acted paradoxically, while the 1 and 10% mediums followed the course to be anticipated. Both the 5 and 10% bile-peptone solutions exhibited distinct germicidal properties in the initial 5 hours, which was followed in the 5% solution by a slow, but progressive proliferation of the remaining viable cells. On the other hand, the 10% solution continued to destroy the inoculated germs until com-

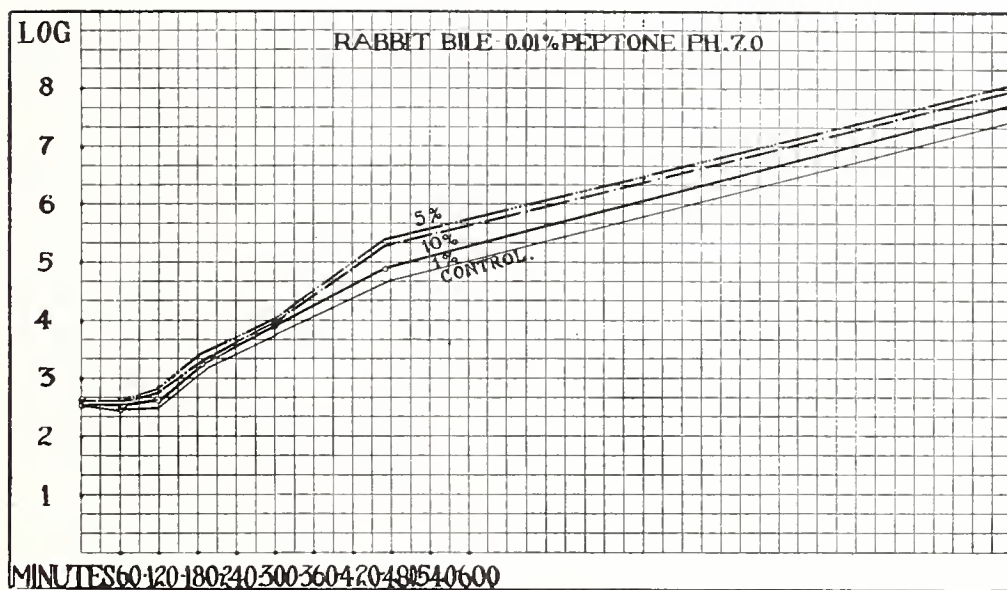


Chart 7.—Rate of Growth of *B. typhosus* in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 7.0.

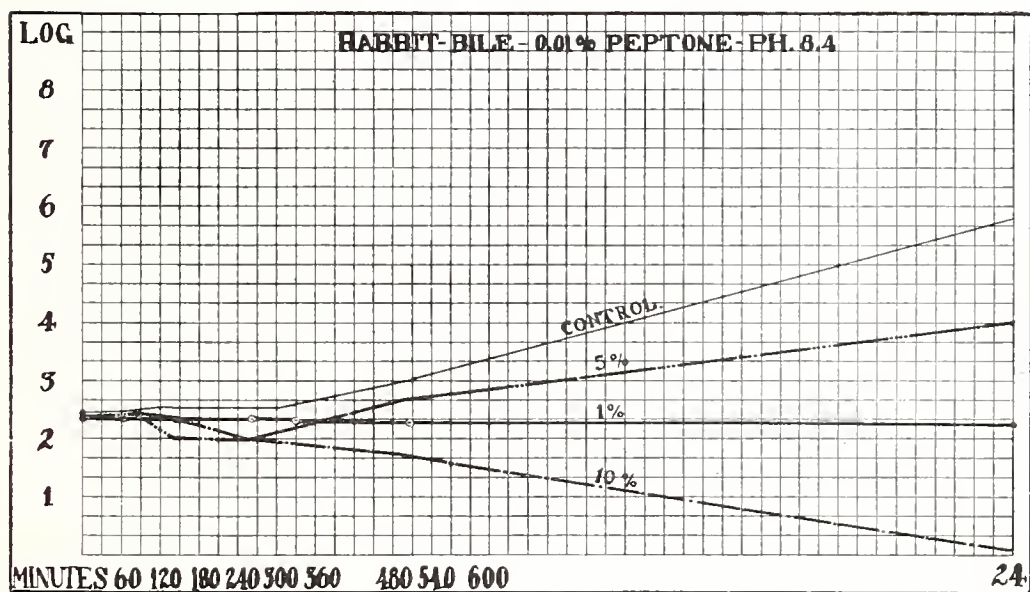


Chart 8.—Rate of growth of *B. typhosus* in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 8.4.

plete sterilization resulted in the 24th hour. The 1% solution exhibited a distinct bacteriostatic action. The available figures indicate that typhoid bacilli either remained alive or were slightly reduced in numbers. At P_H 8.4 rabbit hepatic duct bile added in varying amounts to peptone-phosphate solutions may therefore exhibit three different properties: it maintains growth, it is bacteriostatic, or it is germicidal. The various properties ascribed to animal bile by numerous investigators can obviously be recorded on one and the same specimen. The nature of the inhibitive substances has been considered in the preceding paper. It was concluded that in all probability the bile salts deserve a more detailed investigation. With this conception in mind a series of tests were conducted with pure bile salts.

Exper. 5.—Sodium taurocholate and sodium glycocholate in a 0.01% peptone-phosphate solution were seeded and incubated as usual. Three experiments were made.

A superficial inspection of the curves revealed the same tendency in the rate of growth of *B. typhosus* in pure bile salt mediums as already noted and discussed for the fresh or the desiccated ox bile. The growth of *B. typhosus* was excellent at P_H 7.0, but it was inhibited at P_H 8.4. Moreover, distinct differences existed between the peptone solution containing sodium glycocholate and those prepared with sodium taurocholate. At neutrality sodium glycocholate in 0.5% and 1% solutions was bacteriostatic for 3 hours, but at the end of 24 hours the number of viable cells exceeded those of the control flask. A somewhat similar effect was noted for the taurocholates. From the available data, however, it was impossible to state whether sodium glycocholate was more stimulative at P_H 7.0 than the sodium taurocholate. In comparison with the results obtained at a low H-ion concentration the observation deserves recognition and will repay additional investigation.

The outstanding feature of the action of purified bile salts at P_H 8.2 was the strongly germicidal effect of sodium glycocholate in 0.5% concentration, the inhibitive and slightly antiseptic effect of 1% sodium taurocholate, and the growth-depressing properties of the same salt in 0.5% solutions. It was quite evident that in alkaline mediums glycocholates were considerably more germicidal than the taurocholates, a fact which has hitherto not been emphasized. Meyerstein⁵ noted the absence of growth of *B. typhosus* in a 0.01% Witte's peptone solution

⁵ Centrbl. f. Bakteriöl., I, O., 1904, 44, p. 138.

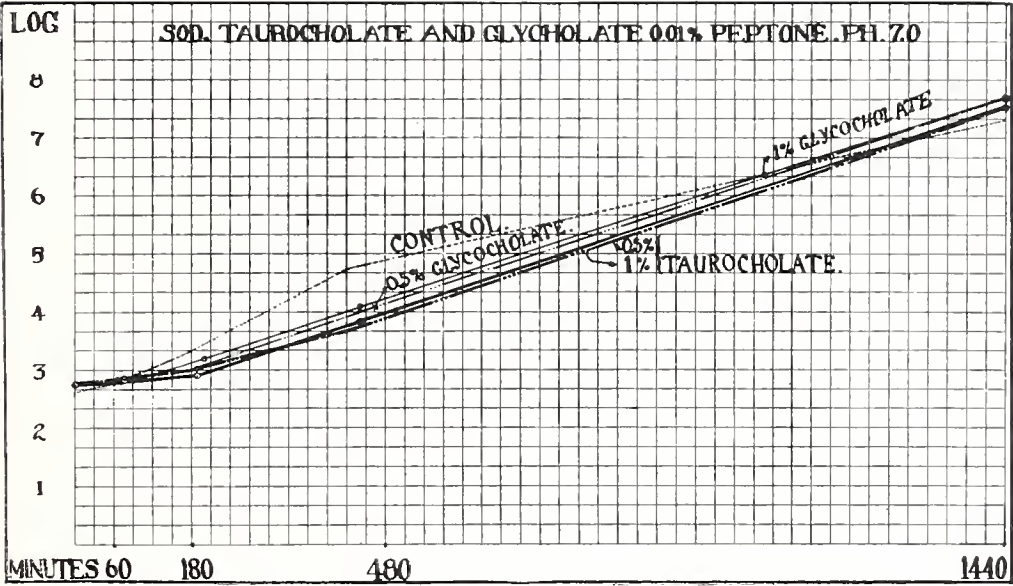


Chart 9.—Rate of growth of *B. typhosus* in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, PH 7.0.

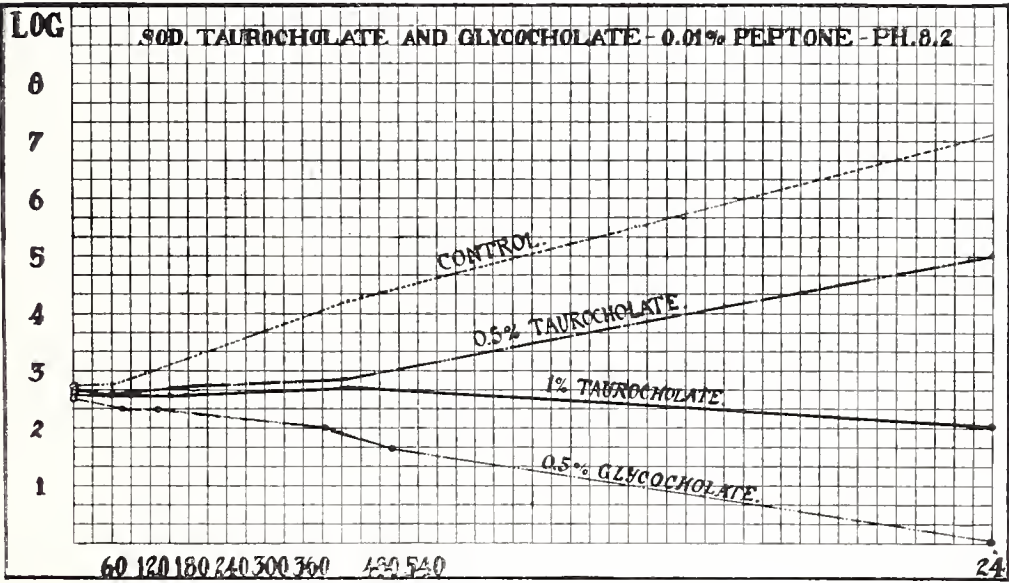


Chart 10.—Rate of growth of *B. typhosus* in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, PH 8.2.

containing 5% sodium glycocholate, while Dünschman⁶ apparently made a similar observation, which prompted him to write the following sentence: "le glycocholate, de son côté agit plutôt à la façon d'un antiseptique."

Even the growth maintaining 0.5% sodium taurocholate-peptone solution was not indifferent to the typhoid bacillus: an 8-hour lag precedes the slow but progressive growth.

DISCUSSION

It is evident from the foregoing data that the rate of growth and the action of the bile and the bile salts of various animals on *B. typhosus* in simple mediums is materially influenced by the reaction. At neutrality small amounts of ox bile, "Bacto" desiccated bile, sodium taurocholate and glycocholates and fairly large amounts of hepatic duct bile of the rabbit are distinctly stimulating. These results are definitely demonstrable when a simple 0.01% peptone-phosphate solution is used as a basic substratum. As a rule, bile concentrations of over 1% are distinctly inhibitive at a P_H 7.0. Rabbit bile in this respect offers an exception for the following reason: It is technically impossible to obtain a sufficient amount of cystic bile to conduct a well-controlled series of tests. The hepatic duct bile employed is less concentrated and contains about $\frac{1}{10}$ of the organic and inorganic elements ordinarily encountered in the gallbladder bile of cattle. It is also possible that rabbit bile is an exceptionally good medium for *B. typhosus* at a neutral or slightly alkaline reaction. Our observations made in vivo and in vitro with cystic bile of these animals and reported in the preceding papers lend some support to this contention.

The nature of the stimulating action has not been investigated. It may be that the bile or bile salts supply only additional food material to a comparatively poor nutritive substratum. The bile and bile salts may contain some of the mysterious substances classified with the so-called "vitamines" that play an important rôle in animal metabolism. Viewed from this standpoint the bacteriologic aspect of bile offers an unlimited number of experimental possibilities.

In an alkaline medium (P_H 8.2 to 8.4) the bile specimens or their salts invariably cause distinct inhibition in concentrations below 0.5%. The addition of comparatively large amounts of the same bile or biliary salts renders the medium not only inhibitive, but directly germicidal.

⁶ Ann. de l'Inst. Pasteur, 1909, 23, p. 48.

In all such mediums the lag is increased, and there may be a distinct bacteriostatic effect for from 5 to 10 hours. A slight initial growth may be followed by a slow, but progressive, destruction of the viable cells. In ox bile the prolonged lag may be replaced by an active growth leading after 24 hours to a number of viable cells nearly as great as the one recorded in the bile-free control tube. This phenomenon is probably the result of adaptation of *B. typhosus* to the antagonistic forces of the alkaline-bile mediums. It is possible that the inhibitive substances, as for example the bile salts, are decomposed as a result of the growth of *B. typhosus* (Exner and Heyrovsky) and transformed into growth-enhancing food elements. Our experiments are not sufficiently numerous, nor have they been planned with this question in mind. The data at our disposal permit one conclusion, namely, that the bile salts, and particularly the glycocholates, are the substances which inhibit or diminish the viable cells of *B. typhosus* in weak peptone solutions. Furthermore, the interesting and noteworthy fact is recorded that the bile salts in the concentration usually present in normal bile exert their germicidal properties when the medium is alkaline. In searching for an analogous condition, the result of studies of several investigators are recalled, namely, certain dyestuffs are more potent in a medium with a reaction in the alkaline range than in one at or near neutrality. Prowazek⁷ and Traube⁸ found that sodium carbonate accentuates the toxicity of methylene blue or of crystal violet. Brown-ing, Gulbranson and Kennway⁹ also obtained remarkable results in the sterilizing effect of diaminoacrididine-methyl-chloride by changing the P_H of the medium from 4.0 to 11.0. Neither of these workers offers a conclusive explanation for this phenomenon, but it is not unlikely that the view of Traube, who found that sodium carbonate produced changes in the surface tension of the dye solutions, is applicable to the bile salts mediums. In this connection the observations of Larson, Cantwell and Hartzell¹⁰ with pneumococci should be mentioned. Bile reduces the surface tension of fluids and, according to the foregoing writers, favors the disintegration of pneumococci. How far our findings are analogous to those just stated must be determined by further tests. One fact is certain: Bile salts are more readily soluble in alkaline solution than in neutral or slightly acid mediums.

⁷ Arch. f. Protist., 1910, 18, p. 221.

⁸ Biochem. Ztschr., 1912, 43, p. 496.

⁹ Jour. Path. & Bacteriol., 1919, 23, p. 106.

¹⁰ Jour. Infect. Dis., 1919, 25, p. 45.

For the present the selective germicidal action of glycocholates in alkaline mediums cannot be explained. An observation of considerable practical importance is that bile and bile salts derived from various animals contain varying amounts of this acid. According to Hammarsten,¹¹ rabbit bile and some samples of ox bile possess exclusively glycocholates; while dog bile is stated to be deprived of this substance. Variation in the cultural properties of bile samples must be ascribed to the composition of the secretion, and not purely to the reaction, as is the case in the observations of Nichols. Chemical analyses of hepatic duct bile of rabbits are not available for comparison, but it is not unlikely that similar unexplicable fluctuation in the bile acid content may occur, as has been so clearly demonstrated by Foster, Hooper and Whipple¹² for the dog. Future bacteriologic studies on bile should therefore appreciate not only the variability of the reaction, but of the composition as well.

In all probability the antiseptic action of glycocholates or taurocholates does not occur in the animal body. A reaction conducive to the development of germicidal properties is found only in the test tube exposed to air, and not in the gallbladder in vivo. This point has been treated in detail in the preceding papers.

As far as the recorded observations have some practical bearing on the use of bile or bile salt mediums, it can be stated that such additions to nutritive mediums will be advantageous only in neutral or slightly acid substratums, and when the concentration does not exceed 1% fresh or 0.5-1% desiccated ox bile. The value of bile additions should never be over-estimated, primarily on account of the comparatively slight stimulating, selective effect on *B. typhosus*, secondarily on account of the production of a distinct lag, which is provoked by fairly small amounts of bile salts. The recommendation of Tonney, Caldwell and Griffin¹³ in disregarding lactose bile for the isolation of *B. typhosus* from the stool is fully justified in the light of our findings.

CONCLUSIONS

Bile of oxen, hepatic duct bile of rabbits, bacto "desiccated ox bile," sodium glycocholate and taurocholate in 1% concentration in a 0.01% "Difco" peptone-phosphate solution at a P_H 7.0 are growth-enhancing

¹¹ *Ergebn. d. Physiologie*, 1905, 4, p. 1.

¹² *Jour. Biol. Chem.*, 1919, 38, p. 379.

¹³ *Jour. Infect. Dis.*, 1916, 18, p. 239.

for *B. typhosus*, while greater amounts, such as 3 to 30%, greatly inhibit proliferation. At P_H 8.4 the same bile specimens or their salts acquire either inhibitive, bacteriostatic or germicidal properties. The more concentrated the mediums are in biliary salts, the greater is their effect on the viable cells. Even small amounts of bile salts, such as 0.5%, destroy the inoculated bacteria in 24 hours. At P_H 8.4 glycocholates are more antiseptic than taurocholates, while the same salts in the same concentration may be stimulative at a P_H 7.0.

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